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Award Number: W81XWH-11-1-0097

TITLE: The Role of Skp1-Cul1-F-box Ubiquitin Ligases in Src-Stimulated Estrogen Receptor Proteolysis and Estrogen Receptor Target Gene Expression

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REPORT DATE: March 2014

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

X Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
March 2014	Annual Summary	01January2011-31December2013

4. TITLE AND SUBTITLE
The Role of Skp1-Cul1-F-box Ubiquitin Ligases in Src-Stimulated Estrogen Receptor Proteolysis and Estrogen Receptor Target Gene Expression

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0097

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Miami

Miami, FL 33136-1002

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research
And Materiel Command
Fort Detrick, Maryland
21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Estrogen triggers transactivation coupled estrogen receptor α (ERα) proteolysis, but mechanisms thereof remain obscure. Present data link estrogen:ERα-driven transcription with cell cycle progression. Here, we identify SKP2 as a late-acting coactivator that drives ERα targets to promote G1-to-S progression. Data support a model in which estrogen-activated ERα phosphorylation, to prime ERα-SCF^{SKP2} binding in late G1. SKP2 activates ERα ubiquitylation and proteolysis. Putative late ERα targets were identified by expression profiling. SKP2 knockdown attenuated E2F-1 and BLM induction. SKP2 overexpression enhanced estrogen-induced E2F-1 and BLM expression. SKP2 knockdown impaired estrogen-stimulated ERα, SKP2, SRC3 and RNA polymerase II recruitment to E2F-1 and BLM promoters. SKP2 serves as dual ERα E3 ligase/coactivator for late-activated target genes, revealing a novel mechanism whereby ERα/SCF^{SKP2} transactivation of E2F-1 feeds forward to drive G1-to-S.

15. SUBJECT TERMS
26S proteasome; Binding (Molecular Function); Breast; Estrogen receptor alpha; Cyclin E-Cdk2; E2F-1; BLM; In vitro; Molecular Target; Phosphorylation; Tumor; Ubiquitination

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USARMRC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	86	**19b. TELEPHONE NUMBER** *(include area code)*
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Introduction

Breast cancer is the most frequent women's cancer, and about 70% of these tumors express estrogen receptor α (ER) (Jordan, 1995). ER is a transcription factor and master regulator of estrogen stimulated proliferation and its expression indicates potential for response to estrogenic stimulation(Bookout et al., 2006). A majority of ER positive breast cancers initially respond well to selective estrogen receptor modulators (such as tamoxifen) or to aromatase inhibitors. Understanding the regulation of ER levels and its role in transcription of estrogen driven genes is thus highly germane to breast cancer therapy(Deroo and Korach, 2006; Chang et al., 2008). The purpose of this Predoctoral Traineeship Award was to support Dr. Wen Zhou's current breast cancer research on understanding the regulation of ERα levels and its role in transcription of estrogen driven genes. Based on the evidence that for many transcription factors, activation is linked to transcription factor degradation and estrogen stimulation is known to activate ERα proteolysis, we hypothesized that **estrogen activated ER ubiquitination may be mediated by the SCF ubiquitin ligases**. Specific AIMs were: **1)** To test if SCF ligases stimulate ER proteolysis in cells; **2)** To test if SCF ligases promote ligand stimulated ER proteolysis in vitro; and **3)** To test if SCF ligases act as ER coactivators in ligand activated ER target gene expression. For the past three years, we have been dissecting the role of ubiquitin E3 ligase SCF^{SKP2} in regulating ER stability and transcriptional activities.

Progress in this area was described in the previous reports, and the data were published in two recent publications, including a publication in **Oncogene** wherein SCF^{SKP2} is recruited when ER is phosphorylated by Cyclin E/CDK2 at S341 (**PMID # 23770852**) and one in **Nature Reviews Cancer** wherein we reviewed the link between receptor degradation coupled activation by different E3 ligases (**PMID # 2450518**). This final report summarizes below all our findings up to date, which support the hypothesis that estrogen triggered E3 ligase recruitments to ER drive receptor proteolysis linked activation. The work summarized in this final report is within the general scope of our approved **SOW** and also complements our original proposal with the supportive data and synergistic extension.

Body

Task 1. To test if SCF E3 ligases involve in Src stimulated estrogen activated ER proteolysis in vivo (Months 1-12).
Task 2. To test if SCF E3 ligase involve in Src stimulated ER proteolysis in vitro (Months 13-24).
Task 3. To test if SCF E3 ligase involve in Src stimulated ER Target Gene Expression and to prepare the manuscript for publication (Months 25-36).

We have fully finished the proposed research in all three tasks and the obtained results have been published in an Oncogene paper entitled "ER, SKP2 and E2F-1 form a feed forward loop driving late ER targets and G1 cell cycle progression " (Appendix I). The F-box protein SKP2 was suggested by recent studies as a proto-oncogene in several primary cancers, including breast cancer. But to our knowledge, a direct association between SKP2 with ER had not been previously reported by the time we carried out our study. We found overexpression of SKP2 in ER positive MCF-7 and ZR-75-1 breast cancer cell lines accelerated liganded ER degradation, while shRNA knockdown SKP2 impaired ER proteolysis. ER phosphor-deficient mutant ER S341A failed to bind SKP2, and was more stable compared to liganded wild-type ER when stably expressed in the ER negative MDA-MB-231 line. Synchronized MCF-7 cells showed ER-CDK2 binding peaked just before ER-SKP2 binding peaked during the G1/S transition. ChIP and sequential ChIP showed ER and SKP2 were involved in the same complex binding to target gene E2F1 and BLM promoters, and RT-qPCR showed shSKP2 impaired these target expressions. All these evidences suggested SKP2 is an ER co-activator as well as ER E3 ligase.

<u>SCF^{SKP2} complex binding to ERα is estrogen dependent</u>
Several F-box proteins contain the signature steroid hormone co-activator motif LXXLL. To test if SCFs regulate ERα proteolysis, a dominant negative CUL1 ($Cul1^{DN}$) was overexpressed in MCF-7 to disrupt SCF function. $Cul1^{DN}$ did not affect ERα levels in the absence of 17-β-estradiol (E2) but reproducibly attenuated E2-triggered ERα degradation (Figure 1A). Since Cul1 is required for SCF-mediated proteolysis, this supported further analysis of LXXLL-bearing F-box proteins as regulators of ER proteolysis (see below).

The F-box protein, SKP2, localizes to the nucleus (Carrano et al., 1999) and contains two LXXLL motifs. As such, it could participate in ligand activated nuclear ERα proteolysis (Reid et al., 2003). Immunoprecipitates of cellular SCFSKP2 components, SKP1, SKP2, CUL1 and RBX1 from two different ERα-positive lines, MCF-7 and ZR-75-1 all contained associated ERα (Figure 1B). None of these proteins bound non-specific antibody. Immunodepletion of SKP2 followed by ERα IP suggests only a minority of ER is detected in SKP2 complexes

In both MCF-7 and ZR-75-1, ERα-SKP2 interaction was not detected in E2-deprived cells but increased rapidly after E2 addition (Figure 1C). Pretreatment with E2 antagonist, 4-hydroxytamoxifen impaired E2-stimulated SKP2-ERα binding (Figure 1C). That ERα-SKP2 binding is estrogen-dependent is not surprising since the coactivator LXXLL-binding surface in ERα is only exposed in the presence of estrogenic ligand and is not available in unliganded or tamoxifen-bound ERα. While a 1 hr pre-treatment with proteasome inhibitor, Z-Leu-Leu-Leu-CHO (MG-132) was too short to significantly affect ERα or SKP2 levels, E2-stimulated ERα-SKP2 complexes were stabilized (Figure 1D), compatible with a transient ERα-SKP2 interaction preceding ERα proteolysis in both lines.

SKP2 mediates estrogen dependent ERα ubiquitylation and degradation in cells and in vitro

To further test if SCF^{SKP2} mediates ERα degradation, three stable SKP2-overexpressing MCF-7 lines were constructed (Figure 2A). Cycloheximide (CHX) chase showed high ERα stability in the absence of E2 both without and with SKP2 overexpression (t1/2> 24 hrs). SKP2 overexpression reduced ERα t1/2 from 6 to 4.5 hours at 2 hrs after E2 addition (Figure 2A), thus SKP2 is rate-limiting for E2-dependent ERα degradation.

Two different SKP2 ShRNA lentiviri (ShSKP2-1 and ShSKP2-2) were used to stably knockdown SKP2 in MCF-7. E2-deprived ShSKP2 and control MCF-7 showed a similar ERα t1/2. In contrast, at 2 hrs after E2 stimulation in both MCF-7 shSKP2 lines, the ERα t1/2 was increased nearly two-fold (ERα t1/2=11 hr) compared to scramble Sh-controls (ERα t1/2=6 hr) (representative data for ShSKP2-1 and ShSKP2-2, Figure 2B). This and the finding that ERα and SKP2 fail to interact in the absence of E2 (Figure 1C), suggest SCF^{SKP2}-mediated ERα degradation is E2 dependent.

To further demonstrate a role for SCF^{SKP2} in ERα proteolysis, enzymatic SCF^{SKP2} complexes were reconstituted in vitro. In vitro ubiquitylation assays with recombinant ERα, ATP, ubiquitin, ubiquitin activating enzyme and hCdc34 showed SCF^{SKP2} ubiquitylates ERα (Figure 2C). Extended reactions with added 26S proteasome showed the SCFSKP2 complex can mediate ERα proteolysis (Figure 2D).

LQTLL (aa 248-252) in SKP2 mediates binding to ERα

Since tamoxifen (which obscures the LXXLL binding site on ERα) disrupts ERα-SKP2 binding (Figure 3-1C), we assayed if SKP2 LXXLL mutations would abrogate ERα-binding. SKP2 has two LXXLL motifs: in its F-box domain (aa 113-118) and in a leucine-rich repeat (aa 248-252). Xpress-tagged SKP2 vectors encoding wild type and mutants at LPEA117A118, LQTA251A252, or both sites--2X LXXAA were stably expressed in MCF-7. WT-SKP2 and SKP2 LPEA117A118 stably bound ERα, but SKP2 LQTA251A252 and the double SKP2 LXXLL-mutant binding to ERα was much reduced (Figure 3A). Thus the LQTL251L252 is important for SKP2:ERα binding. Crystal structure analysis reveals that SKP2 binds SKP1 through LPEL117L118 F-box (Schulman et al., 2000), thus simultaneous SKP2 interaction with both SKP1, and ERα as its F-box substrate would require use of the 248LQTLL252 for ERα binding.

ERαSer341 phosphorylation primes SKP2 binding and ER-ubiquitylation

Several substrates require phosphorylation at a “phosphodegron” for recognition by SKP2 (Glickman and Ciechanover, 2002). The minimal substrate phosphorylation motif required for SKP2 binding is EXS/T (Hao et al., 2005). ERα has two such sequences, EPS137 and EAS341. The S341 site is highly conserved. Since Cyclin E-CDK2 phosphorylates EXS/T sites in other substrates (Hao et al., 2005), we assayed its potential to phosphorylate ERα to prime SKP2 binding.

Wild type ERα (WT-ERα) and mutant ERα proteins (S137A, S341A, S137A/S341A) were overexpressed in ERα-negative MDA-MB-231 cells. WT-ERα and the ERαS137A binding to SKP2 were similar, but ERαS341A and the double mutant, ERαS137A/S341A, both bound SKP2 poorly (Figure 3B).

To characterize further if ERαS341 phosphorylation primes E2-stimulated ERα proteolysis, the stability of WT-ERα and the phospho-deficient ERαS341A mutant were compared by cycloheximide chase in stably transfected MDA-MB-231 lines. E2-stimulated ERαS341A proteolysis was decreased 2-fold compared to WT-ERα (Figure 3C, t1/2 of 12 hrs vs 6 hrs) when CHX was added 2 hrs after E2, consistent with the decrease in ERαS341A:SKP2 binding (Figure 3B).

To test if Cyclin E-CDK2 could phosphorylate the ERα EXS motif, in vitro kinase assays tested eight different ERα wild type or serine to alanine EPS137 or EAS341 mutants peptides as

substrates. S-to-A mutation at each putative site decreased phosphorylation of the respective peptide. The stoichiometry of phosphorylation of the two EAS341 containing peptides supports their highest probability as substrates. Phosphorylation of both ERαEAS341 peptides (long and short forms) by recombinant Cyclin E-CDK2 was four-fold greater than of ERαEPS137 peptides indicating that EAS341 is preferred over EPS137 in vitro (Figure 3D).

Interestingly, comparison of recombinant full-length WT and mutant ERα as Cyclin E-CDK2 substrates in vitro showed strongly attenuated phosphorylation of ERαS341A, but not of ERαS137A (Figure 3E), supporting the importance of S341 to CDK2-dependent ERα phosphorylation. Cyclin E-CDK2 pre-treatment of ERα increased in vitro ubiquitylation (Figure 3F, top) and proteasomal degradation (Figure 3F, bottom) of recombinant WT-ERα and ERαS137A, but not that of ERαS341A.

ERα Ser 294 phosphorylation was recently reported to regulate ERα: SKP2 association (Bhatt et al., 2012). Properties of full-length WT-ERα, ERαS341A and ERαS294A were compared. Both ERα mutants showed similar ability to WT- ERα to drive luciferase reporter expression over 3 logs of E2 (10^{-11} M to 10^{-8} M) when overexpressed in ERα negative HeLa cells, indicating they can bind ligand

WT and mutant ERα proteins were precipitated from transfected HEK 293T lines, and used as substrates in Cyclin E-CDK2 assays, followed by ubiquitylation and proteasomal degradation assays. As in Figure 3-3E, ERα phosphorylation by Cyclin E-CDK2 was significantly reduced by S341A and was also reduced by S294AIn vitro, Cyclin E-CDK2 pretreatment increased WT-ERα ubiquitylation by SCFSKP2. Cyclin E-CDK2 pretreatment did not stimulate ubiquitylation of ERαS341A, which was less than that of WT- ERα was similar to that of WT-ERα both +/- Cyclin E-CDK2 pre-treatment. SCFSKP2 mediated in vitro proteolysis of WT-ERα was stimulated by Cyclin E-CDK2 pre-treatment, while ERαS341A was unaffected by Cyclin E-CDK2 and resistant to proteolysis). Thus, while CDK2-mediated in vitro phosphorylation of ER is attenuated by mutations affecting both S294 and S341, the S341 site constitutes the “phosphodegron” for estrogen-driven ERα and SKP2 association.

SKP2-ERα complex formation is biphasic during estrogen stimulated cell cycle re-entry

Estrogen-deprivation of MCF-7 induces quiescence, and E2 repletion rapidly activates both cell cycle re-entry (Cariou et al., 2000) and ERα proteolysis (Nawaz et al., 1999; Sun et al., 2012). MCF-7 cells were synchronized in G0/G1 by 48 hrs E2 deprivation. Cell cycle profiles after E2 addition showed early S phase entry by 12 hrs, with peak S phase at 21 hrs (Figure 4A, E). Cellular SKP2 levels were minimal in G0/early G1, rising at the G1/S transition (Figure 4A). Cyclin E and CDK2 protein levels were unchanged during G1-to-S phase, but T160-phosphorylated CDK2 increased (Gu et al., 1992) (Figure4A) with Cyclin E-CDK2 activation. Cyclin E-CDK2 activity increased by 8-12 hrs and peaked by 16 hrs, before peak S phase (Figure 4B, E).

Despite the decline in ERα, its co-precipitation with CDK2 increased during G1 (Figure 4C, E). Notably, although ERα levels fell and SKP2 levels increased during G1 to S phase, ERα-SKP2 binding increased in late G1, peaking after Cyclin E-CDK2 activation (Figure 4B, D, E). The kinetics of these events, graphed in Figure 4E, support a model in which activated Cyclin E-CDK2 binds and phosphorylates ERα to prime its recognition by SKP2.

Recent work suggests that ERα phosphorylation at Ser294 by MAPK promotes SKP2 binding (Bhatt et al., 2012). Estrogen rapidly activates MAPK within 5 min, with inactivation by 6 hrs (Figure 3-4F). In early G1, SKP2 t1/2 and levels are low (Wirbelauer et al., 2000) (Figure 4B). Despite low SKP2 levels, ERα-SKP2 complexes were detected 1 hour after E2 (Figure 1C), considerably before Cyclin E-Cdk2 activation. Comparison of early and late time points revealed two phases of ERα-SKP2 binding (Figure 4F). Complexes were absent in estrogen starved cells,

but low ERα-SKP2 complex levels were detected within 5 minutes after E2 stimulation, co-incident with rapid MAPK activation, remained stable between 15 minutes to 6 hrs, then increased dramatically after Cyclin E-CDK2 activation in late G1. When SKP2 levels increased, ER levels were significantly decreased, thus protein levels did not favor binding in late G1.

We next compared effects of CDK2 (Roscovitine) and MEK (U0126) inhibitors. Both blocked E2 stimulated G1- to- S phase progression (Figure 3-4H). UO126 abolished both the rapid E2 driven MAPK activation and early ERα-SKP2 complex assembly, while Roscovitine did not affect either. However, the CDK2 inhibition by Roscovitine abolished the dramatic late G1 rise in ERα-SKP2 complexes. MEK inhibition prevented cyclin E-CDK2 activation, arrested cell cycle progression and both early and late phases of ERα-SKP2 assembly were lost (IP-Blots and quantitation shown in Figure 3-4F-G). These data suggest that both MAPK and CDK2 may promote ERα-SKP2 binding, with the former playing an early role and CDK2 driving late assembly.

In E2 deprived cells, ERα is stable (Alarid et al., 1999). E2 addition rapidly (within minutes) stimulates ER proteolysis (Chu et al., 2007; Sun et al., 2012). Since Cyclin E-CDK2 activation and SKP2 rise in late G1, SKP2 would affect a later phase of E2 activated ERα-degradation. This model would predict that ERα proteolysis kinetics may differ between early and late G1. Cycloheximide chases started at 3 and 12 hrs after estradiol addition, respectively showed ERα t1/2 of 6 hrs in early G1 and a t1/2 of 5 hrs in late G1/S (Figure 4I). This bimodal pattern with different ERα half-lives early and late after estrogen stimulation, suggests early and late mechanisms govern E2-activated receptor proteolysis.

<u>SCF^{SKP2} regulates ERα target gene expression for G1/S transition and S phase progression</u>

If SKP2 acts as ubiquitin ligase for ERα and as co-activator, SKP2-ERα driven gene targets would be induced late in G1, after CyclinE-CDK2 activation. To identify putative SKP2-coactivated ERα targets that increase in late G1/S, MCF-7 gene expression profiles were compared before and early (at 3, 6 hr) or late (12 hrs) after E2 stimulation. Total RNA from triplicate samples was extracted, labeled and hybridized to Agilent whole genome arrays representing >41,000 transcripts (Figure 5A).

Differential expression was assessed as the average ratio between two treatment conditions, with > 2-fold change with a false discovery rate less than 0.05. Late estrogen activated genes were selected using a cutoff of 1.5-fold increase between 6 and 12 hrs. Twenty-two genes were upregulated in late G1 by this criterion (Figure 5B). Many of these are involved in the G1/S transition or mitosis (E2F-1, FBXO5/EMI1), and in DNA replication (BLM, CDC6, RFA). Over 80% of the genes we found increased by >1.5 fold between 6 and 12 hrs after E2 addition were also upregulated by E2 in 3 other publically available array databases (Lin et al., 2007a; Carroll et al., 2006; Lin et al., 2007b).

Of these late E2 activated genes, several contained ERα binding AP-1/Sp-1 sites within 10KB of their promoter start sites and were predicted by the Hormone Receptor Target Database (Kennedy et al., 2010) to be ERα targets. Several also bear partial ERE consensus motifs in their promoters (Figure 5B). QPCR confirmed late upregulation for two of these, E2F-1 and BLM (Figure 5D). E2 stimulated a modest early E2F-1 induction, within 3-6 hrs in both MCF-7 and ZR-75-1, but E2F-1 mRNA levels rose, significantly by 12 hrs (Figure 5D & F).

In both MCF-7 and ZR-75-1, SKP2 knockdown delayed and attenuated peak S phase entry (Figures 5C& E) and decreased the late induction of E2F-1 and/or BLM (Figure 5D & F). Notably, the early E2F-1 induction by estrogen was not affected by SKP2 knockdown, but its late G1 upregulation was. Not all E2 driven genes expressed late were SKP2-regulated: the late E2-activated RAB31 induction was not affected by SKP2-knockdown. Canonical ERα target genes,

such as pS2 and GREB1 were rapidly activated by E2-stimulation, but were not affected by SKP2-knockdown.

WT-SKP2 overexpressing MCF-7 showed a notable increase in late E2-mediated E2F-1 and BLM induction at 12 and 24 hrs after E2 addition (Figure 5G, H); this was not seen in cells overexpressing the C-terminal LXXLL mutant, SKP2-LQTA251A252. SKP2-LPEA117A118 did not differ from SKP2 WT in its effects on E2F-1 and BLM activation.

SCF^{SKP2} binds late ERα target gene promoters

E2F-1 and BLM are known to be upregulated by ERα/Sp-1 or ERα/AP-1 binding (Iso et al., 2007; Wang et al., 1999). To further investigate whether SKP2 coactivates ERα at these target genes, SKP2 and ERα binding to their Sp-1/AP-1 promoter elements was assayed by ChIP. ERα occupied both E2F-1 (Figure 6A and B) and BLM (Figure 6C) promoters late after E2 stimulation. Binding increased by between 12-18 hrs and was inhibited by tamoxifen. In both MCF-7 and ZR-75-1, ChIP/re-IP showed late E2-stimulated E2F-1 promoter co-occupancy by ERα, SKP2, SRC-3 and RNA polymerase II (PolII) that was blocked by tamoxifen pre-treatment with (Figure 6A and B). Similar findings were observed for BLM (Figure 6C).

Finally, Xpress-WT-SKP2, but not the LXXLL mutant SKP2-L248QTA251A252, showed a considerable increase in binding at E2F-1 and BLM promoters at 12 and 24 hrs after estrogen stimulation (Figure 6D). Binding of the more proximal LXXLL mutant SKP2-LPEA117A118 to the respective E2F-1 and BLM promoter Sp-1/AP-1 elements did not differ significantly from WT-SKP2. Taken together, these data suggest that E2F-1 and BLM are part of a subset of late-activated ERα target genes co-activated by SKP2.

Key Research Accomplishments

1) SCF^{SKP2} regulates ERα protein stability
   a) $Cul1^{DN}$ transfection impairs ERα degradation
   b) ERα is present in Skp1-SKP2-Cul1-Rbx1 complex
   c) Proteasome inhibitor MG-132 stabilizes SKP2-ERα binding
   d) Overexpression of SKP2 accelerates E2 stimulated ERα proteolysis
   e) SKP2 shRNA delays E2 stimulated ERα proteolysis

2) SCF^{SKP2} mediates ERα ubiquitination in vitro and mapping ERα-SKP2 binding motifs
   a) Cellular SCF^{SKP2} ubiquitinates ERα in vitro
   b) Reconstituted SCF^{SKP2} ubiquitinates and degrades ERα in vitro
   c) LXXLL in SKP2 mediates ERα/SKP2 binding to promote ERα loss
   d) SKP2 LXXLL mutant LQTA251A252 failed to bind ERα
   e) ERα EXS mutant S341A is more stable than wild type ERα
   a) ERSer341 phosphorylation primes SKP2 binding and ER ubiquitylation

3) ER, SKP2 and E2F-1 form a feed forward loop driving late ER targets and G1 cell cycle progression
   a) SKP2-ER complex formation is biphasic during estrogen-stimulated cell cycle re-entry
   b) SCF^{SKP2} regulates ER target gene expression for G1/S transition and S-phase progression
   c) SCF^{SKP2} binds late ER target gene promoters

Reportable Outcome

1) Peer-reviewed paper

a) Zhou W, Slingerland JM. Links between steroid receptor activation and proteolysis: potential relevance to therapy of hormone regulated cancers. Nature Revs Cancer. 2014 Jan;14(1):26-38. (Appendix 2)

b) Zhou W, Srinivasan S, Nawaz Z, Slingerland JM. ER, SKP2 and E2F-1 form a feed forward loop driving late ER targets and G1 cell cycle progression. Oncogene. 2013 Jun 17. doi: 10.1038/onc.2013.197. (Appendix 1)

2) Abstract accepted by conferences

a) Zhou W, Srinivasan S, Nawaz Z, Slingerland JM. ER, SKP2 and E2F-1 form a feed forward loop driving late ER targets and G1 cell cycle progression. Annual Zubrod Memorial Lecture and Cancer Research Poster Session, May 17, 2013, Miami, FL. (Appendix 4)

b) Zhou W, Slingerland JM. The SCF F box protein, SKP2, is a novel estrogen receptor α dual-role coactivator that affects cancer cell progression. Nature Biotech Miami 2013 Winter Symposium "The Molecular Basis of Metabolism and Nutrition", February 10-13, 2013, Miami, FL. (Appendix 5)

c) Zhou W, Sun J, Srinivasan S, Nawaz Z, Slingerland JM. SCF/SKP2 E3 ligase promotes G1/S transition by ubiqutinating and activating estrogen receptor α. FASEB Summer Research Conference “The Physiology of Intergrated Nuclear and Extranuclear Steroid Signalling”, July 29-August 3, 2012, Snowmass Village, CO. (Appendix 6)

d) Zhou W, Sun J, Srinivasan S, Nawaz Z, Slingerland JM. The SCF F Box Protein, SKP2, is a Key Component of an E3 Ubiqutin Ligase that Governs Estrogen Receptor α Stability. Annual Zubrod Memorial Lecture and Cancer Research Poster Session, May 18, 2012, Miami, FL. (Appendix 7)

e) Zhou W, Sun J, Slingerland JM. The SCF F box Protein, SKP2, is a key component of an E3 ubiqutin ligase that governs estrogen receptor α stability. In: Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research; 2012 Mar 31-Apr 4; Chicago, IL. Cancer Res 2012;72: 952. (Appendix 8)

3) Oral presentations to discuss the outcome of this grant

a) **Zhou W.** The biochemical characterization of SKP1-CUL1-RBX1-SKP2 complex as estrogen receptor co-activator-E3 ligase. **Rockefeller University.** December 19, **2013**, New York, NY. (Invited talk)

b) **Zhou W.** The characterization of SCF^{SKP2} functions on ER both as E3 ligase and coactivator. **Columbia University.** December 17, **2013**, New York, NY. (Invited talk)

c) **Zhou W**. ER, SKP2 and E2F-1 form a feed forward loop driving late ER targets and G1 cell cycle progression. **Children Hospital of Philadelphia** (CHOP) Abramson Cancer Center. October 9, **2013**, Philadelphia, PA. (Invited talk)

d) **Zhou W**. ER, SKP2 and E2F-1 form a feed forward loop driving late ER targets and G1 cell cycle progression. **University of Pennsylvania** Smilow Translational Research Center. October 7, **2013**, Philadelphia, PA. (Invited talk)

e) **Zhou W**, Sun J, Kaliappan K, Nawaz Z, Slingerland JM. The roles of E6AP and SCF (Skp1•CUL1•F-box) in Src stimulated, estrogen activated ER proteolysis and transactivation. **FASEB Summer Research Conference** "The Physiology of Intergrated Nuclear and Extranuclear Steroid Signalling", August 8-13, **2010**, Snowmass Village, CO.

4) Book Chapter

Sun J, Zhou W, Nawaz Z and Slingerland JM. (2012) Cross Talk Between ER and Src Signaling and Its Relevance to ER Status and Hormone Responsiveness.In "Advances in Rapid Sex-Steroid Action".G. Castoria and A. Migliaccio (eds.), Chapter DOI: 10.1007/978-1-4614-1764-4_4, Springer Science+Business Media, LLC. Book Print ISBN 978-1-4614-1763-7. eISBN: 9781461417644. ePub date: Dec 15, 2011. (Appendix 3)

5) Degree obtained and employment opportunity based on training supported on this grant

Obtained PhD (**Jan 2014**) from the work described above and I have already accepted a position for a postdoctoral fellow at Dr. Carol Prives laboratory, Columbia University, New York, NY. I will start my new position in September, 2014.

Conclusion

Here we report that SCF^{SKP2}, comprised of SKP2, in association with SKP1, CUL1 and RBX1, is a ubiquitin E3 ligase for ERα. Ectopic expression of dominant negative Cul1 increases ERα levels by impeding ERα degradation in breast cancer cells. Knockdown of SKP2 impairs estrogen-triggered ERα proteolysis, while ectopic SKP2 expression decreased ERα stability. We show that SKP2, SKP1, RBX1 and CUL1 co-precipitate with cellular ERα and the formation of this ERα/SCF^{SKP2} complex is cell cycle regulated and parallels CDK2 activation. We also show ERα is an in vitro substrate that is ubiquitylated and degraded by SCF^{SKP2}. CDK2-dependent ERα phosphorylation primed ERα/SCF^{SKP2} binding and ERα proteolysis and the subsequent transcriptional activation of *E2F-1* and *BLM*. These data suggest that SKP2 plays an important role in the regulation of ERα stability and transcriptional activity in breast cancer model.

Our study indicated that SKP2 mediated both ER activation and proteolysis. It supports a model that phosphorylation dependent ER-ubiquitylation may modify the conformation of ER-coactivators complexes to drive both ER's transcriptional activation on target genes and drive ER degradation. The proper degradation of ER during or immediately after the transcriptional activation of target genes is essential for ER transcriptional activity. The work elucidating novel molecular mechanisms linking ER proteolysis and activation of ER target genes. The relevance to breast cancer is potentially very significant and is reported in our Nature Reviews Cancer (see also Appendix 2).

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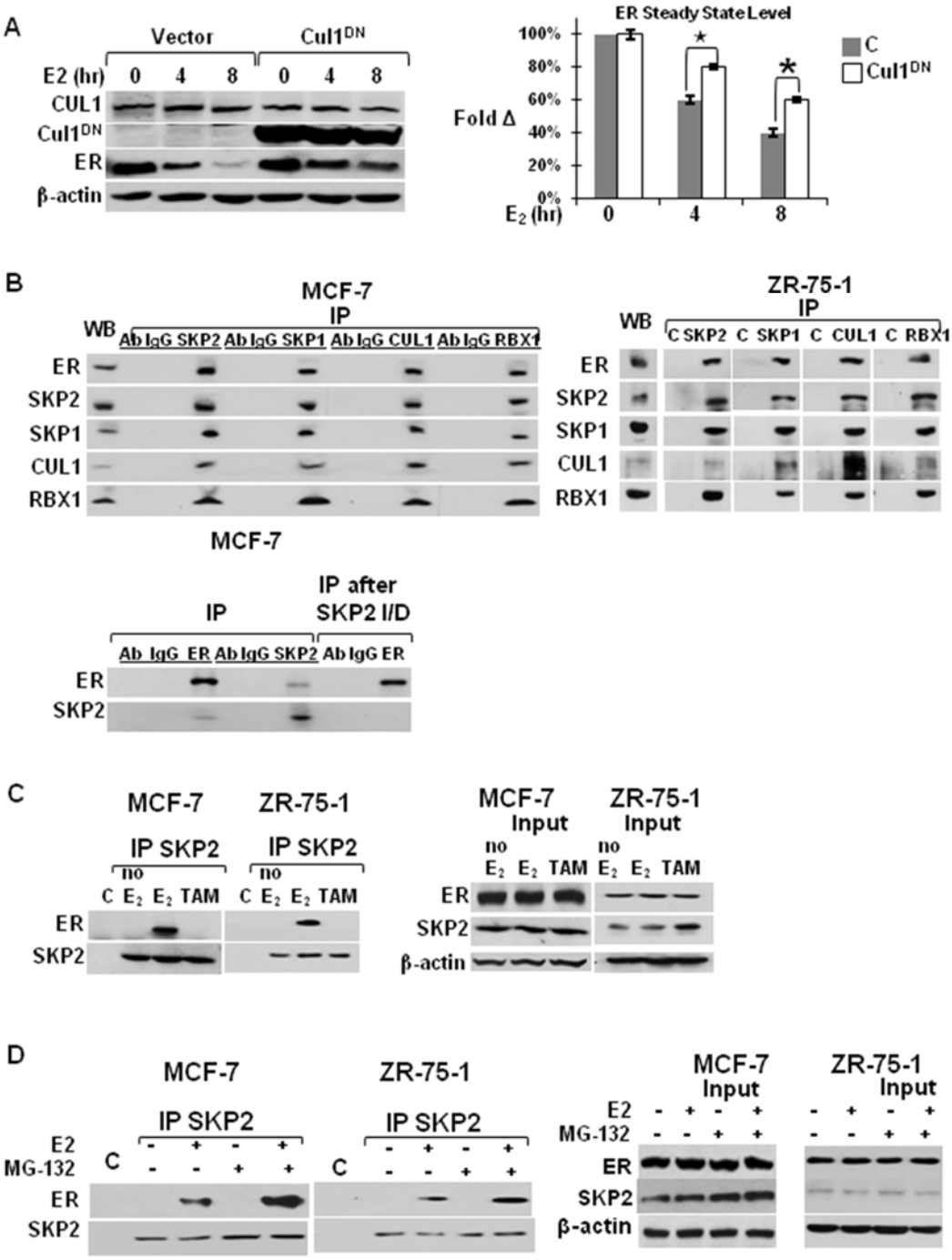
Appendices

Figures and figures legends

Figure 1. **SCF^{SKP2} binds ER on estrogen stimulation.** (a) MCF-7 cells were transfected with vector (c) or Cul1DN, E2-deprived 48 h then treated with 10^{-8} M E2 for 4 or 8 h. Western blots show ER and CUL1, with b-actin loading control. Data from three independent experiments performed in triplicate were plotted as percentage of remaining ER level (±s.e.m.) relative to ER level at the time of E2 depletion. Significance was determined using a two-tailed Student's t-test (★ or *P<0.05). (b) Immunoprecipitations (IP) of indicated SCF^{SKP2} components were performed using 1mg total cell lysate and associated proteins detected by blotting in MCF-7 (left) and ZR-75-1 cells (right). Antibody without lysate served as control (Ab or C). Nonspecific polyclonal antibody failed to precipitate these proteins (immunoglobulin G). To estimate the amount of ER associated with SKP2, cell lysates were immunoprecipitated with either anti-ER antibody, with anti-SKP2 antibody or with anti-ER antibody after three serial immunodepletions with anti-SKP2-conjugated beads (see lower panel). (c) E2-deprived MCF-7 and ZR-75-1 cells were treated with vehicle (no E2), 10^{-8}M E2, or 10^{-5}M 4-hydroxytamoxifen (TAM) for 1 h. Lysates were assayed for SKP2-bound ERa (left, lane 1 is IP control as above). Right panel shows input on western blot. (d) E2-deprived MCF-7 and ZR-75-1 were treated ±E2 for 1 h, ±MG-132 addition immediately before E2, lysed and assayed for SKP2-bound ERa. Lane 1 is control as above. Right panel shows input on western blot.


A
Vector
Cul1DN
E2 (hr) 0 4 8 0 4 8
CUL1
Cul1DN
ER
β-actin
ER Steady State Level
100%
80%
60%
40%
20%
0%
Fold Δ
E2 (hr) 0 4 8
C
Cul1DN
B
MCF-7
IP
WB
Ab IgG SKP2 Ab IgG SKP1 Ab IgG CUL1 Ab IgG RBX1
ER
SKP2
SKP1
CUL1
RBX1
ZR-75-1
IP
WB
C SKP2 C SKP1 C CUL1 C RBX1
ER
SKP2
SKP1
CUL1
RBX1
MCF-7
IP
IP after SKP2 I/D
Ab IgG ER Ab IgG SKP2 Ab IgG ER
ER
SKP2
C
MCF-7
ZR-75-1
IP SKP2
IP SKP2
no
C E2 E2 TAM C E2 E2 TAM
ER
SKP2
MCF-7
Input
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Input
no
E2 E2 TAM no E2 E2 TAM
ER
SKP2
β-actin
D
MCF-7
ZR-75-1
IP SKP2
IP SKP2
E2
MG-132
C
ER
SKP2
MCF-7
Input
ZR-75-1
Input
E2
MG-132
ER
SKP2
β-actin

Figure 2. **SKP2 levels alter ER stability and SCF^{SKP2} stimulates ER ubiquitylation and proteolysis in cells and *in vitro*.** (a) MCF-7 was stably transfected with empty vector (c) or SKP2 then E2 deprived (E2-) or treated with E2 for 2 h (E2+) followed by addition of CHX and ER assayed by western blot at intervals shown (top). Western blot shows SKP2 with anti-SKP2 or anti-Xpress-tag antibody (middle). ER decay was assayed by densitometry, and mean data from three independent experiments performed in triplicate were plotted as semi-log values relative to ER steady-state level at the time CHX addition. (bottom). Two hours after E2 addition, ER has a $t_{1/2}$ of 6 h in controls, and a $t_{1/2}$ of 4.5 h in MCF-SKP2 (mean±s.e.m.). (b) MCF-7 cells were infected with scramble shRNA (c) or one of two different SKP2 shRNA lentiviri (shSKP2-1, 2). Stable lines were E2 deprived then treated with vehicle (E2-) or E2 for 2 h then CHX added and ER assayed by western blot at intervals shown (top). ERa decay was assayed by densitometry, and data from three independent experiments performed in triplicate plotted as semi-log values as in (a). Western blot shows SKP2 knockdown (lower panel). -Actin serves as loading control. ER t1/2 is 6 h in E2 stimulated controls, and $t_{1/2}$=11 h with stable SKP2 knockdown (mean±s.e.m.). (c) For ER ubiquitylation in vitro, ER, ATP, ubiquitin, E1 and His-hCdc34 were incubated with SCF^{SKP2} and ER precipitates blotted with anti-ubiquitin. (d) In vitro degradation assay was as in (c) with the addition of 26S proteasome complex for the indicated times followed by western blot for ER. Data from three independent experiments performed in triplicate were plotted as percent of remaining ERa protein level (±s.e.m.) relative to ER protein level at the starting time. In lane 6, ER levels were significantly lower at 1 and 2 h compared with T¼0 controls two-tailed Student's t-test (★ and * signify $P<0.05$).

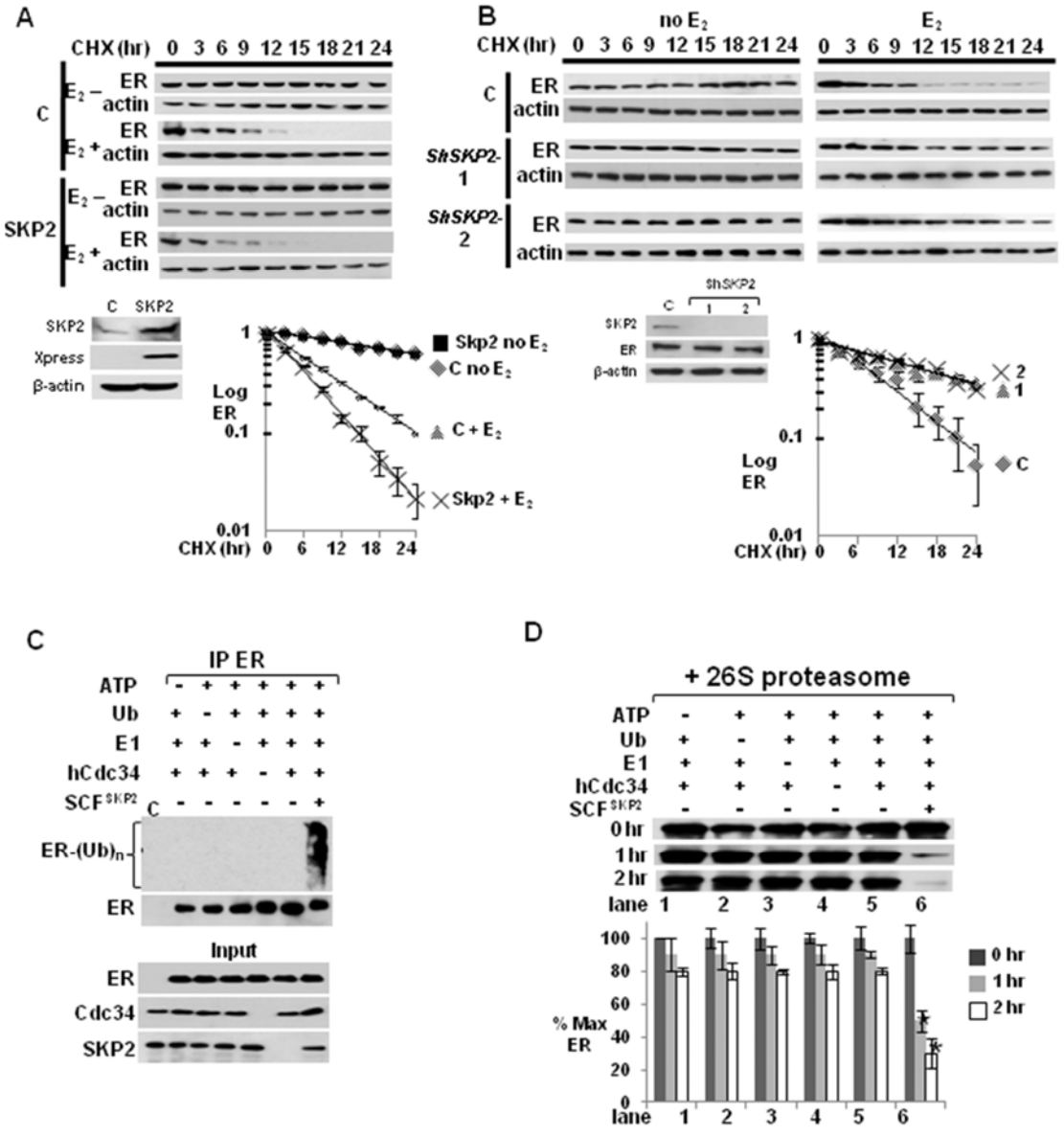

A
CHX (hr) 0 3 6 9 12 15 18 21 24
C
E2 – ER
actin
E2 + ER
actin
SKP2
E2 – ER
actin
E2 + ER
actin
C SKP2
SKP2
Xpress
β-actin
Log ER
1
0.1
0.01
CHX (hr) 0 6 12 18 24
Skp2 no E2
C no E2
C + E2
Skp2 + E2
B
no E2
E2
CHX (hr) 0 3 6 9 12 15 18 21 24
C
ER
actin
ShSKP2-1
ER
actin
ShSKP2-2
ER
actin
C ShSKP2
1 2
SKP2
ER
β-actin
Log ER
1
0.1
0.01
CHX (hr) 0 6 12 18 24
2
1
C
C
IP ER
ATP - + + + + +
Ub + - + + + +
E1 + + - + + +
hCdc34 + + + - + +
SCF SKP2 - - - - - +
C
ER-(Ub)n
ER
Input
ER
Cdc34
SKP2
D
+ 26S proteasome
ATP - + + + + +
Ub + - + + + +
E1 + + - + + +
hCdc34 + + + - + +
SCF SKP2 - - - - - +
0 hr
1 hr
2 hr
lane 1 2 3 4 5 6
% Max ER
100
80
60
40
20
0
lane 1 2 3 4 5 6
0 hr
1 hr
2 hr

Figure 3. SKP2 L**248QTLL252 motif is critical for ER-SKP2 binding, and ER S341 phosphorylation by cyclin E-CDK2 primes ER binding and degradation by SCF^{SKP2} in vitro.** (a) Sequence alignment of LXXLL motifs from SRC1 and SKP2 on top. MCF-7 stably transfected with vector, WT Xpress-SKP2 or Xpress-SKP2 mutants (SKP2 L114PEAA118, SKP2 L248QTAA252 or SKP2-2X LXXAA) were assayed for Xpess-SKP2-bound ER by IP-blot. Antibody without lysate served as control. (b) Sequence alignment of EXT/S motifs from p27, p21 and ER on top. BER-negative MDA-MB-231 cells stably transfected with vector only (vector), WT-ER (ERa WT) or ER mutants (S137A, S341A or S137A-S341A) were assayed for SKP2-bound ER. Antibody without lysate served as control. (c) Stably transfected MDA-MB-231 ER WT and ER S341A were E2 deprived, treated with vehicle (E2-) or estradiol (E2+) for 2 h then CHX added and ER assayed by western blot at intervals shown. -Actin serves as loading control. Densitometry and linear regression of data from three independent experiments performed in triplicate shows ER WT has a $t_{1/2}$ of 6 h, and ER S341A has a $t_{1/2}$ of 12 h (mean±s.e.m.). (d) ER WT and mutant peptides were reacted with cyclin E/CDK2 kinase in vitro. Radioactivity in ER peptides was quantitated by liquid scintillation; data were normalized to highest read, and graphed as means±s.e.m. from triplicate assays. Insert (right) shows control radioactivity recovered on the filter when substrate was omitted from the reaction. Significance was determined by two-tailed Student's t-test by comparing S341 containing peptides with S137 containing peptides (comparison were done for both long forms and short forms) ($^{\star}P<0.05$ or $^{\star\star}P<0.01$). (e) Recombinant FLAG-ER WT, ER S137A, ER S341A and ER S137A-S341A were used as substrate for in vitro kinase. Coomassie staining shows equal input of each purified protein. Control (c) reactions contained all reagents except substrate. Activity of recombinant ER proteins was quantitated by liquid scintillation counting; data were normalized to highest read, and graphed as means±s.e.m. from triplicate assays. Significance was determined using a two-tailed Student's t-test by comparing radioactivity in WT recombinant ER protein with that in ER mutants (* or $^{*}P<0.05$). (f) Recombinant ER WT, ER S137A, ERa S341A and ER S137A-S341A were used as substrate for in vitro ubiquitylation and degradation assays with or without cyclin E/CDK2 pre-treatment.

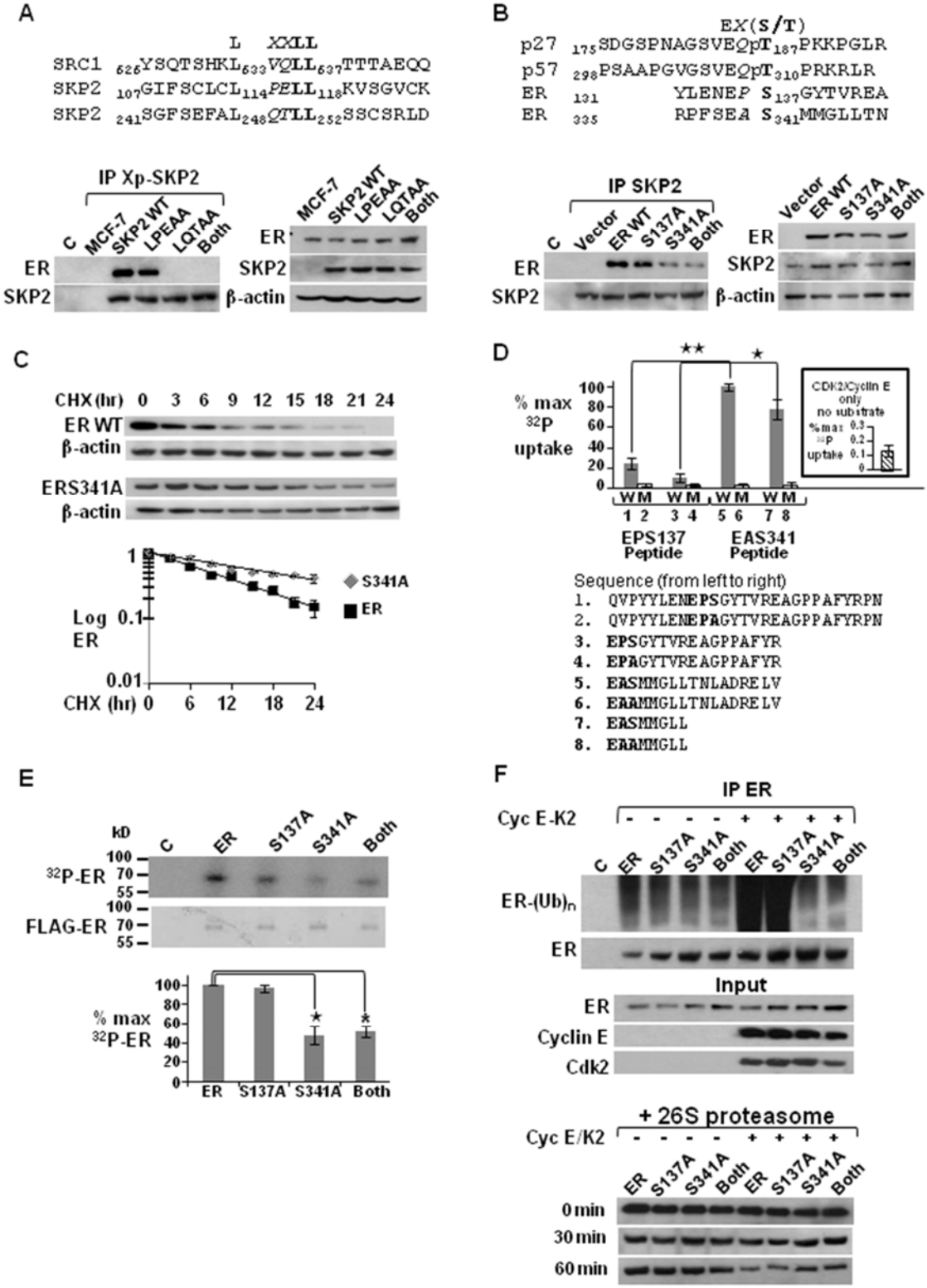

A
L XXLL
SRC1 626YSQTSHKL633VQLL637TTTAEQQ
SKP2 107GIFSCLCL114PELL118KVSGVCK
SKP2 241SGFSEFAL248QTLL252SSCSRLD
IP Xp-SKP2
C
MCF-7
SKP2 WT
LPEAA
LQTAA
Both
ER
SKP2
β-actin
B
EX(S/T)
p27 175SDGSPNAGSVEQpT187PKKPGLR
p57 298PSAAPGVGSVEQpT310PRKRLR
ER 131 YLENEP S137GYTVREA
ER 335 RPFSEA S341MMGLLTN
IP SKP2
Vector
ER WT
S137A
S341A
Both
C
ER
SKP2
β-actin
D
% max 32P uptake
CDK2/Cyclin E only no substrate
WM WM WM WM
1 2 3 4 5 6 7 8
EPS137 Peptide
EAS341 Peptide
Sequence (from left to right)
1. QVPYYLENEPSGYTVREAGPPAFYRPN
2. QVPYYLENEPAGYTVREAGPPAFYRPN
3. EPSGYTVREAGPPAFYR
4. EPAGYTVREAGPPAFYR
5. EASMMGLLTNLADRELV
6. EAAMMGLLTNLADRELV
7. EASMMGL
8. EAAMMGL
C
CHX (hr) 0 3 6 9 12 15 18 21 24
ER WT
β-actin
ERS341A
β-actin
Log ER
S341A
ER
CHX (hr) 0 6 12 18 24
E
kD
C
ER
S137A
S341A
Both
32P-ER
FLAG-ER
% max 32P-ER
ER S137A S341A Both
F
IP ER
Cyc E-K2
ER-(Ub)n
ER
Input
ER
Cyclin E
Cdk2
+ 26S proteasome
Cyc E/K2
0 min
30 min
60 min

Figure 4. **Quantitation of cyclin E-CDK2 activity, CDK2-bound and SKP2-bound ER and ER $t_{1/2}$ during G1-to-S-phase progression.** (a–e) E2-deprived MCF-7 were treated with 10^{-8} M E2 and recovered at intervals shown. (a) Flow cytometry showed cell cycle re-entry (% S phase), and western blots for ER, cyclin E, SKP2 and CDK2 are shown. -Actin serves as loading control. (b) For cyclin E-CDK2 kinase activity, cyclin E was precipitated and reacted with histone H1 and radioactivity in substrate (^{32}P-H1) shown by autoradiography. Activity was quantitated by phospho-imager and graphed as mean % max activity±s.e.m. from >3 assays in (e) below. (c, d) CDK2-bound ER (c) and SKP2-bound ER (d) assayed at intervals after E2 addition. (e) Quantitation of data from a–c above. The experiments were repeated at least three times. Data were normalized to highest read, and graphed as mean % max±s.e.m. from >3 assays. (f) E2-deprived MCF-7 were treated with E2 for intervals shown, lysed and assayed for SKP2-bound ER. Lanes 1 and 2 are antibody control and normal immunoglobulin G control for IP. Lower panel shows input on western blot. (g) SKP2-bound ER was assayed at intervals after E2 addition ±pre-treatment with CDK2 inhibitor Roscovitine or MEK inhibitor U0126, and graphed as mean % max±s.e.m. from >3 assays. Significance was determined using a two-tailed Student's t-test by comparing control with that from drug pre-treatment (★ $P<0.05$ or ** $P<0.01$). (h) Flow cytometry (% S phase) showed cell cycle re-entry at 18 h in control but not in drug treated cells. (i) E2-deprived MCF-7 were treated with E2 for 3 or 12 h before adding CHX and harvested at various time points for ER western blot. -Actin serves as loading control. After 3 h of E2, ER has a $t_{1/2}$ of 6 h, whereas after 12 h of E2, ER has a $t_{1/2}$ of 5 h.

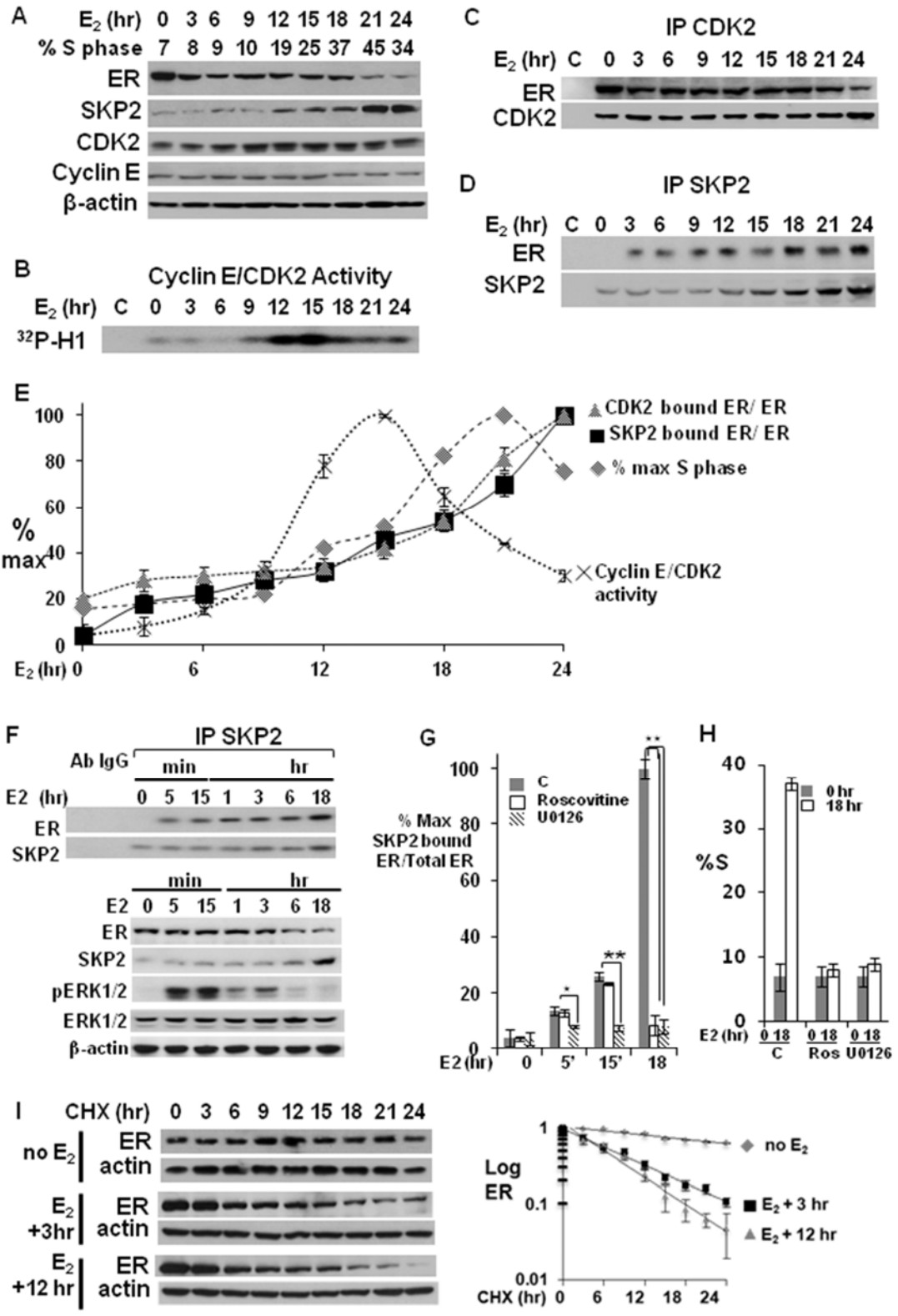

A
E2 (hr) 0 3 6 9 12 15 18 21 24
% S phase 7 8 9 10 19 25 37 45 34
ER
SKP2
CDK2
Cyclin E
β-actin
B
Cyclin E/CDK2 Activity
E2 (hr) C 0 3 6 9 12 15 18 21 24
32P-H1
C
IP CDK2
E2 (hr) C 0 3 6 9 12 15 18 21 24
ER
CDK2
D
IP SKP2
E2 (hr) C 0 3 6 9 12 15 18 21 24
ER
SKP2
E
% max
CDK2 bound ER/ ER
SKP2 bound ER/ ER
% max S phase
Cyclin E/CDK2 activity
E2 (hr) 0 6 12 18 24
F
IP SKP2
Ab IgG
min hr
E2 (hr) 0 5 15 1 3 6 18
ER
SKP2
min hr
E2 0 5 15 1 3 6 18
ER
SKP2
pERK1/2
ERK1/2
β-actin
G
% Max SKP2 bound ER/Total ER
C
Roscovitine
U0126
E2 (hr) 0 5' 15' 18
H
%S
0 hr
18 hr
E2 (hr) 0 18 0 18 0 18
C Ros U0126
I
CHX (hr) 0 3 6 9 12 15 18 21 24
no E2 ER actin
E2 +3hr ER actin
E2 +12 hr ER actin
Log ER
no E2
E2 + 3 hr
E2 + 12 hr
CHX (hr) 0 6 12 18 24

Figure 5. **E2F-1, BLM are part of a subset of late ER-activated genes regulated by SCF^{SKP2}.** (a) Heatmap of E2-stimulated gene expression. Clustering of genes expressed in E2-deprived MCF-7 cells treated with 10^{-8} M E2 for 3, 6, 12 h compared with untreated. (b) Plot of eight ERE-bearing genes whose expression rises >1.5X between 6 and 12 h after E2 stimulation. (c, d) E2-deprived parental MCF-7 or MCF-7 SKP2 knockdown (MCF-7/shSKP2) cells were treated with E2 and assayed at intervals for: (c) Cell cycle profile by flow cytometry, graphed as mean % S-phase cells/time in MCF-7±ShSKP2. (d) E2F-1, BLM and RAB31 transcripts quantitated by reverse-transcription q-PCR. GAPDH was used as an internal control. Data from three biologic experiments are plotted as fold induction over control (mean±s.e.m.). (e, f) Cell cycle profiles (e) and q-PCR for E2F-1 transcript quantitation (f) were carried out as (c, d) in a second ER-positive cell line ZR-75-1 and derivative ZR-75-1/shSKP2. (g, h) MCF-7 transfected with either WT-SKP2, SKP2-$L_{114}PEAA_{118}$ or SKP2-$L_{248}QTAA_{252}$ were E2 deprived and recovered 12 and 24 h after E2 addition for q-PCR of E2F-1 (e) and BLM (f). Significant differences were determined using a two-tailed Student's t-test comparing target gene mRNA levels with WT SKP2 overexpression with that of control cells or cells overexpressing SKP2 mutant $L_{248}QTLL_{252}$ ($\star$ and *P<0.05).

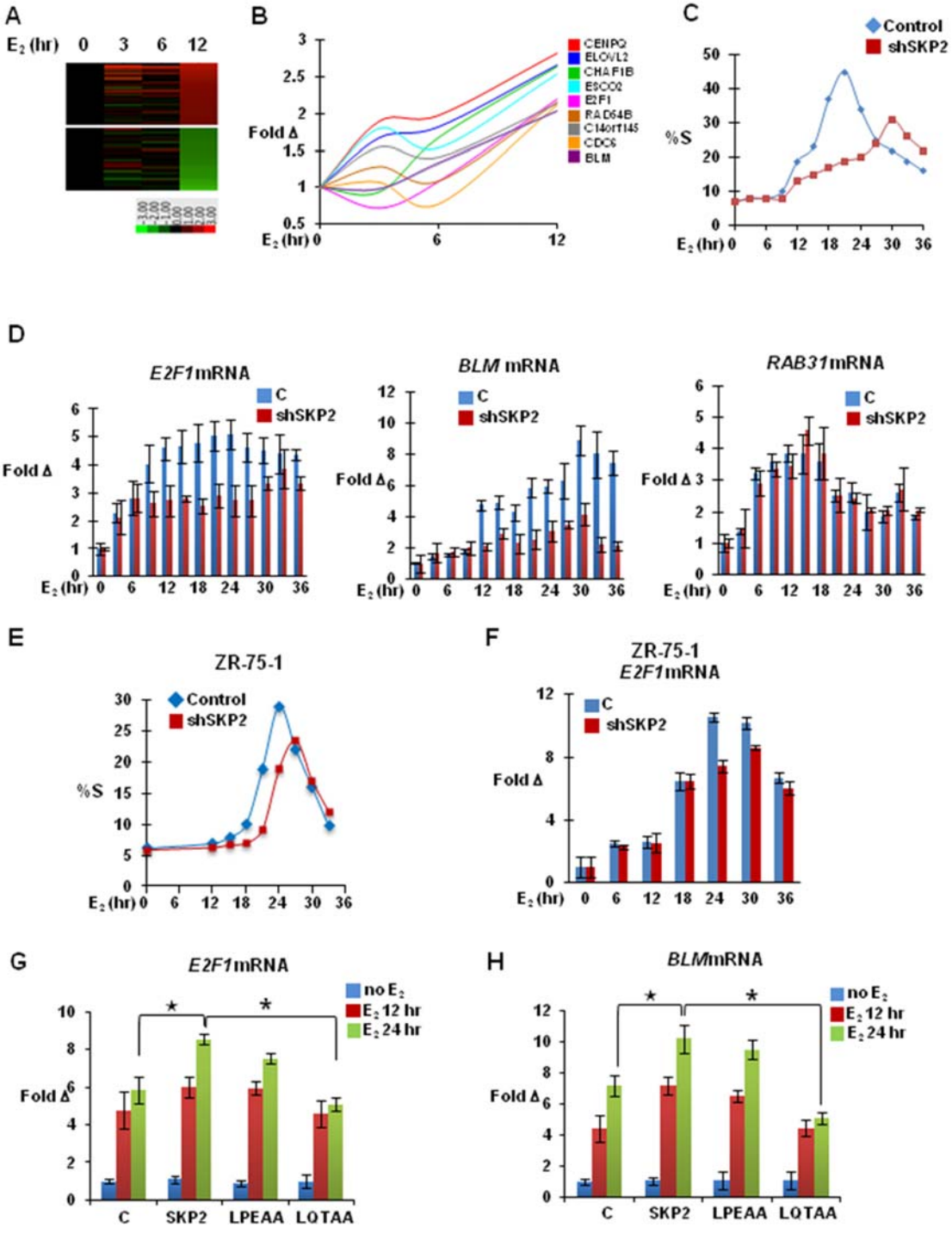
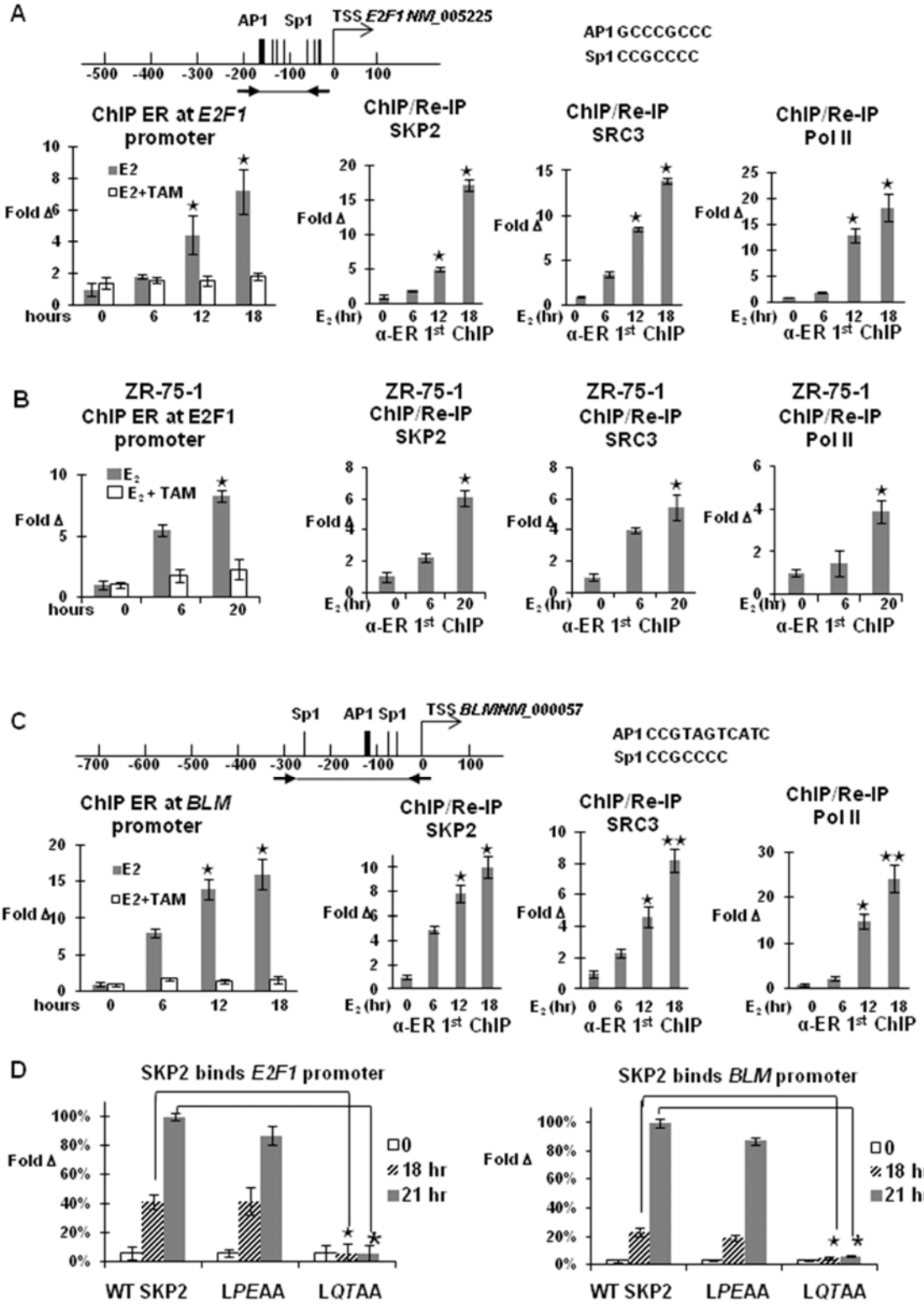

A
E2 (hr) 0 3 6 12
B
Fold Δ
E2 (hr) 0 6 12
CENPQ
ELOVL2
CHAF1B
ESCO2
E2F1
RAD54B
C14orf145
CDC6
BLM
C
Control
shSKP2
%S
E2 (hr) 0 6 12 18 24 30 36
D
E2F1mRNA
BLM mRNA
RAB31mRNA
C
shSKP2
Fold Δ
E2 (hr) 0 6 12 18 24 30 36
E
ZR-75-1
Control
shSKP2
%S
E2 (hr) 0 6 12 18 24 30 36
F
ZR-75-1
E2F1mRNA
C
shSKP2
Fold Δ
E2 (hr) 0 6 12 18 24 30 36
G
E2F1mRNA
no E2
E2 12 hr
E2 24 hr
Fold Δ
C
SKP2
LPEAA
LQTAA
H
BLMmRNA
no E2
E2 12 hr
E2 24 hr
Fold Δ
C
SKP2
LPEAA
LQTAA

Figure 6. SKP**2 directly regulates late ER-activated genes E2F-1 and BLM.** MCF-7 or ZR-75-1 cells were harvested after 48-h E2 deprivation at time=0 h or after 10 nM E2 for the times indicated. (a, b) ER ChIP was performed at the E2F-1 promoter at indicated times in MCF-7 (a) or ZR-75-1 (b). ER ChIP/Re-IP used SKP2, SRC3 or polymerase II (Pol II) Abs for the re-precipitation. The fractions of protein bound to the promoter at different E2 treatment intervals were compared with that of E2 depleted cells and significant differences were determined using a two-tailed Student's t-test ($^{\star}P<0.05$). (c) ER ChIP was performed at the BLM promoter. ER ChIP/Re-IP used antibodies to SKP2, SRC3 or Pol II. The fractions of protein bound to the promoter at different E2 treatment intervals were compared with that of E2 depleted cells and significant differences were determined using a two-tailed Student's t-test ($^{\star}P<0.05$ and $^{\star\star}P<0.01$). (d) MCF-7 cells stably expressing Xpress-tagged WT SKP2, SKP2-L114PEAA118 or SKP2-L248QTAA252 were harvested after 48 h E2 deprivation at time¼0 h or after 10 nM E2 treatment for 18 or 21 h. ChIP experiments were performed at E2F-1 or BLM promoters using anti-Xpress antibody. Significance was determined using a two-tailed Student's t-test by compared the fraction of WT-SKP2 protein binding to gene promoter at 18, 21 h of E2 with that of cells expressing SKP2 L248QTLL252 (*, $^{*}P<0.05$).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D
SKP2 binds E2F1 promoter
SKP2 binds BLM promoter
Fold Δ
0
18 hr
21 hr
WT SKP2
LPEAA
LQTAA

Additional Appendices summary

Appendix 1 Oncogene 2013 reprint

Zhou W, Srinivasan S, Nawaz Z, Slingerland JM. ER, SKP2 and E2F-1 form a feed forward loop driving late ER targets and G1 cell cycle progression. Oncogene. 2013 Jun 17. doi: 10.1038/onc.2013.197. (Epub). (Times Cited: 2)

Appendix 2 Nature Reviews Cancer 2014 reprint

Zhou W, Slingerland JM. Links between steroid receptor activation and proteolysis: potential relevance to therapy of hormone regulated cancers. Nature Revs Cancer. (2013 Nov 11 accepted and arranged for Jan 2014 issue)

Appendix 3 Book Chapter 2012

Sun J, Zhou W, Nawaz Z and Slingerland JM. (2012) Cross Talk Between ER and Src Signaling and Its Relevance to ER Status and Hormone Responsiveness.In "Advances in Rapid Sex-Steroid Action".G. Castoria and A. Migliaccio (eds.), Chapter DOI: 10.1007/978-1-4614-1764-4_4, Springer Science+Business Media, LLC. Book ISBN 978-1-4614-1763-7.

Appendix 4 Zubrod abstract 2013

Zhou W, Srinivasan S, Nawaz Z, Slingerland JM. ER, SKP2 and E2F-1 form a feed forward loop driving late ER targets and G1 cell cycle progression. Annual Zubrod Memorial Lecture and Cancer Research Poster Session, May 17, 2013, Miami, FL.

Appendix 5 Nature Miami 2013 Winter Symposium abstract

Zhou W, Slingerland JM. The SCF F box protein, SKP2, is a novel estrogen receptor α dual-role coactivator that affects cancer cell progression. Nature Biotech Miami 2013 Winter Symposium "The Molecular Basis of Metabolism and Nutrition", February 10-13, 2013, Miami, FL.

Appendix 6 FASEB 2012 abstract

Zhou W, Sun J, Srinivasan S, Nawaz Z, Slingerland JM. SCF/SKP2 E3 ligase promotes G1/S transition by ubiqutinating and activating estrogen receptor α. FASEB Summer Research Conference "The Physiology of Intergrated Nuclear and Extranuclear Steroid Signalling", July 29-August 3, 2012, Snowmass Village, CO.

Appendix 7 Zubrod 2012 abstract

Zhou W, Sun J, Srinivasan S, Nawaz Z, Slingerland JM. The SCF F Box Protein, SKP2, is a Key Component of an E3 Ubiqutin Ligase that Governs Estrogen Receptor α Stability. Annual Zubrod Memorial Lecture and Cancer Research Poster Session, May 18, 2012, Miami, FL.

Appendix 8 AACR 2012 abstract

Zhou W, Sun J, Slingerland JM. The SCF F box Protein, SKP2, is a key component of an E3 ubiqutin ligase that governs estrogen receptor α stability. In: Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research; 2012 Mar 31-Apr 4; Chicago, IL. Cancer Res 2012;72: 952.

Appendix 9 Curriculum Vitae

Appendix 1 Oncogene 2013 reprint

Oncogene (2013), 1–13

www.nature.com/onc

ORIGINAL ARTICLE

ERα, SKP2 and E2F-1 form a feed forward loop driving late ERα targets and G1 cell cycle progression

W Zhou[1,2], S Srinivasan[1,2], Z Nawaz[1,2] and JM Slingerland[1,2,3]

Estrogen triggers transactivation coupled estrogen receptor α (ERα) proteolysis, but mechanisms thereof remain obscure. Present data link estrogen:ERα-driven transcription with cell cycle progression. Although liganded ERα induces many genes within 1–4 h, gene activation after 6 h is thought to be indirect. Here, we identify SKP2 as a late-acting coactivator that drives ERα targets to promote G1-to-S progression. Data support a model in which estrogen-activated cyclin E-CDK2 binds and phosphorylates ERαS341, to prime ERα-SCF^{SKP2} binding via SKP2-L$_{248}$*QT*LL$_{252}$ in late G1. SKP2 activates ERα ubiquitylation and proteolysis. Putative late ERα targets were identified by expression profiling. *SKP2* knockdown attenuated *E2F-1* and *BLM* induction. SKP2 overexpression, but not coactivator motif mutant SKP2-L$_{248}$QTAA$_{252}$, enhanced estrogen-induced *E2F-1* and *BLM* expression. *SKP2* knockdown impaired estrogen-stimulated ERα, SKP2, SRC3 and RNA polymerase II recruitment to *E2F-1* and *BLM* promoters. This work not only identifies these late-activated genes as *bona fide* ERα targets but describes a novel mechanism for their periodic activation. SKP2 serves as dual ERα E3 ligase/coactivator for late-activated target genes, revealing a novel mechanism whereby ERα/SCF^{SKP2} transactivation of *E2F-1* feeds forward to drive G1-to-S.

Oncogene advance online publication, 17 June 2013; doi:10.1038/onc.2013.197

Keywords: cyclin E-CDK2; ERα; E2F-1; SCF^{SKP2}; ubiquitylation; coactivator

INTRODUCTION

Estrogen acts as a cell-type-dependent mitogen by rapid cross talk, activating Src and mitogen-activated protein kinase (MAPK) signaling,[1] and by triggering estrogen receptor α (ERα) to bind coactivators/chromatin remodeling factors to induce mitogenic target gene transcription[2,3] and cell cycle progression.[4] Most ERα coactivators contain one or more Leu-XX-Leu-Leu (L*XX*LL) motifs, which bind directly to ERα helix-12.[5]

Ligand binding to many nuclear hormone receptors, including estrogen,[6,7] thyroid hormone[8] and progesterone[9] receptors activates receptor proteolysis. Mechanisms governing ligand-activated ERα proteolysis are not fully elucidated and may be linked to target gene expression. Coactivator binding may regulate both transcriptional activity and ligand-mediated ERα proteolysis. In some[10,11] but not all models,[12,13] proteasome inhibition decreases estrogen-ERα transcriptional activity despite an increase in ERα abundance. Proteasome inhibitors reduce ERα transcription activity and ultimately re-localize receptor to nucleosomes.[11] Thus, for a subset of ERα-driven genes, ERα ubiquitylation and transcriptional activity may be linked. Indeed, ERα proteolysis after promoter firing may allow promoter re-loading and globally regulate both ERα abundance and overall activity. Constitutive ERα activation could potentially reduce ERα levels via ERα proteolysis.

Signaling pathways that activate many transcription factors (TFs), including c-Jun and c-Myc, also trigger their ubiquitin-dependent degradation,[14] thereby limiting transcriptional activity. TF ubiquitylation may affect coactivator/repressor binding with coactivators enhancing TF ubiquitylation.[14] Ubiquitin is first linked to an ubiquitin-activating enzyme, transferred to an ubiquitin-conjugating enzyme (Ubc), then an ubiquitin ligase, or E3, facilitates substrate ubiquitylation. Substrate polyubiquitylation signals its 26S proteasomal degradation.[15] Certain ERα coactivators have dual roles as E3 ligases. These include E6-associated protein (E6-AP)[16] and murine double minute 2 (MDM2).[17] For many E3 ligases, substrate recognition is regulated by its phosphorylation. Thus, ERα phosphorylation by different signaling pathways could theoretically promote recruitment of different dual role coactivators, thereby changing both the profiles of ERα targets expressed and ERα proteolysis rates.

To elucidate how ERα proteolysis may be linked to target gene expression, we investigated an SCF E3 ligase as putative ERα coactivator. SCF complexes comprise a large E3 family that include SKP1, CUL1 (mammalian homolog of yeast Cdc53), an F-box protein, and RBX1/RBX2 (ROC1/ROC2). Over 70 human F-box proteins bind appropriately phosphorylated substrates to recruit them to the SCF.[18] An *in silico* search revealed several F box proteins contain the ERα coactivator signature motif, L*XX*LL. One of these, SKP2, mediates degradation of several cell cycle proteins.

The present study links ERα transcriptional activity with the cell cycle machinery. Cell cycle progression is governed by cyclin-dependent kinases (CDKs). In G1, cyclin D-bound CDK activation precedes cyclin E-CDK2 activation and both coordinately phosphorylate the retinoblastoma protein to activate the E2F-1 TF.[19] E2F-1, in turn, transactivates genes required for S-phase progression. Many cyclin E-CDK2 substrates promote S and G2/M progression.[19,20]

Here, we identify SCF^{SKP2} as an E3 ligase involved in estrogen-activated ERα degradation and present a model in which cyclin

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Received 26 October 2012; revised 28 March 2013; accepted 12 April 2013

E-CDK2 binds and phosphorylates ERαS341 to prime ERα interaction with SKP2 in late G1. Microarray analysis identified putative late upregulated ERα-target genes. Two late-G1 ERα targets, *E2F-1* and *BLM*, are co-regulated by SKP2, supporting the notion that SCF^{SKP2} has a dual role as E3 ligase and coactivator for ERα. Data also reveal a feed forward loop whereby estrogen stimulates SKP2-dependent ERα transactivation of *E2F-1*, which in turn induces further *cyclin E* and *SKP2* expression to drive S-phase entry.

RESULTS

SCF^{SKP2} complex binding to ERα is estrogen dependent

As noted above, several F-box proteins contain the signature steroid hormone coactivator motif L*XX*LL. To test if SCFs regulate ERα proteolysis, a dominant-negative CUL1 ($Cul1^{DN}$) was overexpressed in MCF-7 to disrupt SCF function. $Cul1^{DN}$ did not affect ERα levels in the absence of 17-β-estradiol (E2) but reproducibly attenuated E2-triggered ERα degradation (Figure 1a). As CUL1 is required for SCF-mediated proteolysis, this supported further analysis of L*XX*LL-bearing F-box proteins as regulators of ERα proteolysis (see below).

The F-box protein, SKP2, localizes to the nucleus[21] and contains two L*XX*LL motifs. As such, it could participate in ligand-activated nuclear ERα proteolysis.[11] Immunoprecipitates of cellular SCF^{SKP2} components, SKP1, SKP2, CUL1 and RBX1 from two different ERα-positive lines, MCF-7 and ZR-75-1 all contained associated ERα (Figure 1b). None of these proteins bound nonspecific antibody. Immunodepletion of SKP2 followed by ERα IP suggests only a minority of ERα is detected in SKP2 complexes (Figure 1b, lower panel).

In both MCF-7 and ZR-75-1, ERα-SKP2 interaction was not detected in E2-deprived cells but increased rapidly after E2 addition (Figure 1c, see also Figures 4d and f). Pre-treatment with E2 antagonist, 4-hydroxytamoxifen prevented E2-stimulated SKP2-ERα binding (Figure 1c). That ERα-SKP2 binding is estrogen-dependent is not surprising as the coactivator L*XX*LL-binding surface in ERα is only exposed in the presence of estrogenic ligand and is not available in unliganded or tamoxifen-bound ERα. Although a 1-h pre-treatment with proteasome inhibitor, Z-Leu-Leu-Leu-CHO (MG-132) was too short to significantly affect ERα or SKP2 levels, E2-stimulated ERα-SKP2 complexes were stabilized (Figure 1d), compatible with a transient ERα–SKP2 interaction preceding ERα proteolysis in both lines.

SKP2 mediates estrogen-dependent ERα ubiquitylation and degradation in cells and *in vitro*

To further test if SCF^{SKP2} mediates ERα degradation, a stable SKP2-overexpressing MCF-7 line was constructed (Figure 2a). Cycloheximide (CHX) chase showed high ERα stability in the absence of E2 both without and with *SKP2* overexpression ($t_{1/2} > 24$ h). E2 driven ERα proteolysis increased with *SKP2* overexpression, with the ERα $t_{1/2}$ falling from 6 to 4.5 h (Figure 2a); thus SKP2 is rate limiting for E2-dependent ERα degradation.

Two different *SKP2* ShRNA lentiviri (ShSKP2-1 and ShSKP2-2) were used to stably knockdown SKP2 in MCF-7. E2-deprived ShSKP2 and control MCF-7 showed a similar ERα $t_{1/2}$. In contrast, after E2 stimulation in both MCF-7 shSKP2 lines, the ERα $t_{1/2}$ was increased nearly twofold (ERα $t_{1/2} = 11$ h) compared with scramble Sh-controls (ERα $t_{1/2} = 6$ h; representative data for ShSKP2-1 and ShSKP2-2, Figure 2b). This and the finding that ERα and SKP2 fail to interact in the absence of E2 (Figure 1c), suggest SCF^{SKP2}-mediated ERα degradation is E2 dependent.

To further demonstrate a role for SCF^{SKP2} in ERα proteolysis, enzymatic SCF^{SKP2} complexes were reconstituted *in vitro*. *In vitro* ubiquitylation assays with recombinant ERα, ATP, ubiquitin, ubiquitin-activating enzyme and hCdc34 showed SCF^{SKP2} ubiquitylates ERα (Figure 2c). Extended reactions with added 26S proteasome showed the SCF^{SKP2} complex promotes ERα proteolysis (Figure 2d).

LQTLL (aa 248–252) in SKP2 mediates binding to ERα

Since tamoxifen which obscures the L*XX*LL binding site on ERα, disrupts ERα-SKP2 binding (Figure 1c), we assayed if mutations that disrupt SKP2 L*XX*LL would abrogate ERα-binding. SKP2 has two L*XX*LL motifs: in its F-box domain (aa 113–118) and in a leucine-rich repeat (aa 248–252). Xpress-tagged SKP2 vectors encoding wild type (WT) and mutants at $L_{114}PEAA_{118}$, $L_{248}QTAA_{252}$, or both sites—2X L*XX*AA were stably expressed in MCF-7. WT-SKP2 and SKP2 $L_{114}PEAA_{118}$ stably bound ERα, but SKP2 $L_{248}QTAA_{252}$ and the double SKP2 L*XX*LL-mutant binding to ERα was much reduced (Figure 3a). Thus, the $L_{248}QTLL_{252}$ is important for SKP2-ERα binding. Crystal structure analysis reveals that SKP2 binds SKP1 through $L_{114}PELL_{118}$ F-box,[22] thus simultaneous SKP2 interaction with both SKP1 and ERα as its F-box substrate would require use of the $L_{248}QTLL_{252}$ for ERα binding.

ERαSer341 phosphorylation primes SKP2 binding and ERα ubiquitylation

Several substrates require phosphorylation at a 'phosphodegron' for recognition by SKP2.[15] The minimal substrate phosphorylation motif required for SKP2 binding is E*X*S/T.[23] ERα has two such sequences, EPS_{137} and EAS_{341}. The S341 site is highly conserved (see Supplementary Figure S1). As cyclin E-CDK2 phosphorylates E*X*S/T sites in other substrates,[23] we assayed its potential to phosphorylate ERα to prime SKP2 binding.

Wild-type ERα (WT-ERα) and mutant ERα proteins (S137A, S341A, S137A/S341A) were overexpressed in ERα-negative MDA-MB-231 cells. WT-ERα and the ERαS137A binding to SKP2 were similar, but ERαS341A and the double mutant, ERαS137A/S341A, both bound SKP2 poorly (Figure 3b).

To characterize further if ERαS341 phosphorylation primes E2-stimulated ERα proteolysis, the stability of WT-ERα and the phospho-deficient ERαS341A mutant were compared by CHX chase in stably transfected MDA-MB-231 lines. E2-stimulated ERαS341A proteolysis was decreased twofold compared with WT-ERα (Figure 3c, $t_{1/2}$ of 12 vs 6 h), consistent with the decrease in ERαS341A-SKP2 binding (Figure 3b).

To test if cyclin E-CDK2 could phosphorylate the ERα E*X*S motif, *in vitro* kinase assays tested eight different ERα WT or serine to alanine E*P*S137 or E*A*S341 mutant peptides as substrates. S-to-A mutation at each putative site decreased phosphorylation of the respective peptide. The stoichiometry of phosphorylation of the two EAS_{341} containing peptides supports their highest probability as substrates. Phosphorylation of both ERαEAS_{341} peptides (long and short forms) by recombinant cyclin E-CDK2 was fourfold greater than of ERαEPS_{137} peptides indicating that EAS_{341} is preferred over EPS_{137} *in vitro* (Figure 3d).

Interestingly, comparison of recombinant full-length WT and mutant ERα as cyclin E-CDK2 substrates *in vitro* showed phosphorylation of ERαS341A was markedly reduced, while that of ERαS137A was not (Figure 3e), supporting the importance of S341 to CDK2-dependent ERα phosphorylation. Cyclin E-CDK2 pre-treatment of ERα increased *in vitro* ubiquitylation (Figure 3f, top) and proteasomal degradation (Figure 3f, bottom) of recombinant WT-ERα and ERαS137A, but not that of ERαS341A.

ERα S294 phosphorylation was recently reported to regulate ERα-SKP2 association.[24] Properties of full-length WT-ERα, ERαS341A and ERαS294A were compared. Both ERα mutants showed a similar ability to WT-ERα to drive luciferase reporter expression over four logs of E2 (10^{-11} to 10^{-8} M) when overexpressed in ERα-negative HeLa cells (Supplementary Figure S2A), indicating they can both bind ligand.

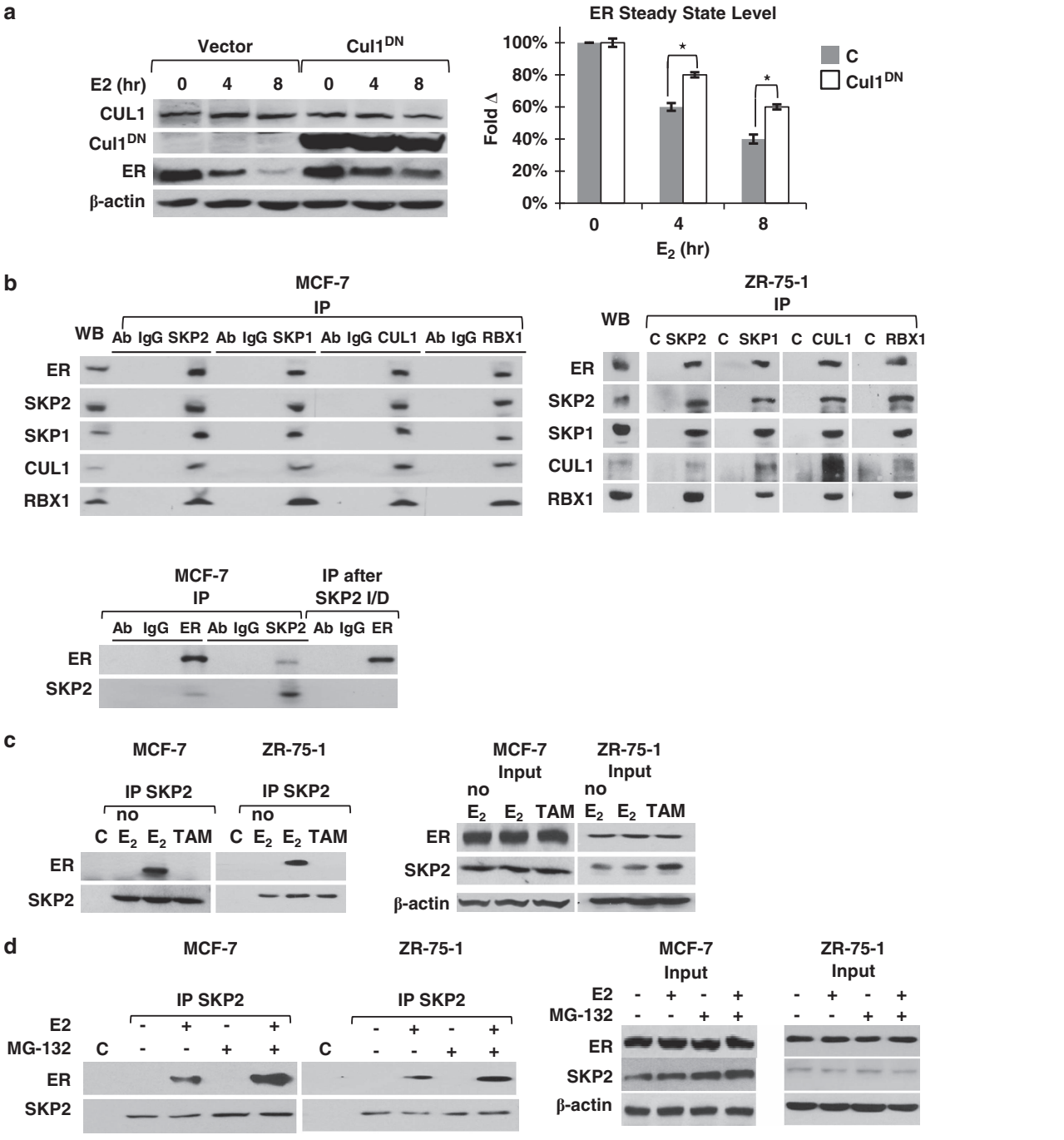

Figure 1. SCF^{SKP2} binds ERα on estrogen stimulation. (**a**) MCF-7 cells were transfected with vector (**c**) or $Cul1^{DN}$, E2-deprived 48 h then treated with 10^{-8} M E2 for 4 or 8 h. Western blots show ERα and CUL1, with β-actin loading control. Data from three independent experiments performed in triplicate were plotted as percentage of remaining ERα level (± s.e.m.) relative to ERα level at the time of E2 depletion. Significance was determined using a two-tailed Student's *t*-test (★ or $*P<0.05$). (**b**) Immunoprecipitations (IP) of indicated SCF^{SKP2} components were performed using 1 mg total cell lysate and associated proteins detected by blotting in MCF-7 (left) and ZR-75-1 cells (right). Antibody without lysate served as control (Ab or C). Nonspecific polyclonal antibody failed to precipitate these proteins (immunoglobulin G). To estimate the amount of ERα associated with SKP2, cell lysates were immunoprecipitated with either anti-ERα antibody, with anti-SKP2 antibody or with anti-ERα antibody after three serial immunodepletions with anti-SKP2-conjugated beads (see lower panel). (**c**) E2-deprived MCF-7 and ZR-75-1 cells were treated with vehicle (no E2), 10^{-8} M E2, or 10^{-5} M 4-hydroxytamoxifen (TAM) for 1 h. Lysates were assayed for SKP2-bound ERα (left, lane 1 is IP control as above). Right panel shows input on western blot. (**d**) E2-deprived MCF-7 and ZR-75-1 were treated ± E2 for 1 h, ± MG-132 addition immediately before E2, lysed and assayed for SKP2-bound ERα. Lane 1 is control as above. Right panel shows input on western blot.

WT and mutant ERα proteins were precipitated from transfected HEK 293T lines, and used as substrates in cyclin E-CDK2 assays, followed by ubiquitylation and proteasomal degradation assays. As in Figure 3e, ERα phosphorylation by cyclin E-CDK2 was significantly reduced by mutations yielding ERαS341A and ERαS294A (Supplementary Figure S2B). *In vitro,* cyclin E-CDK2 pre-treatment increased WT-ERα ubiquitination by SCF^{SKP2}. Cyclin E-CDK2 pre-treatment did not stimulate ubiquitylation of

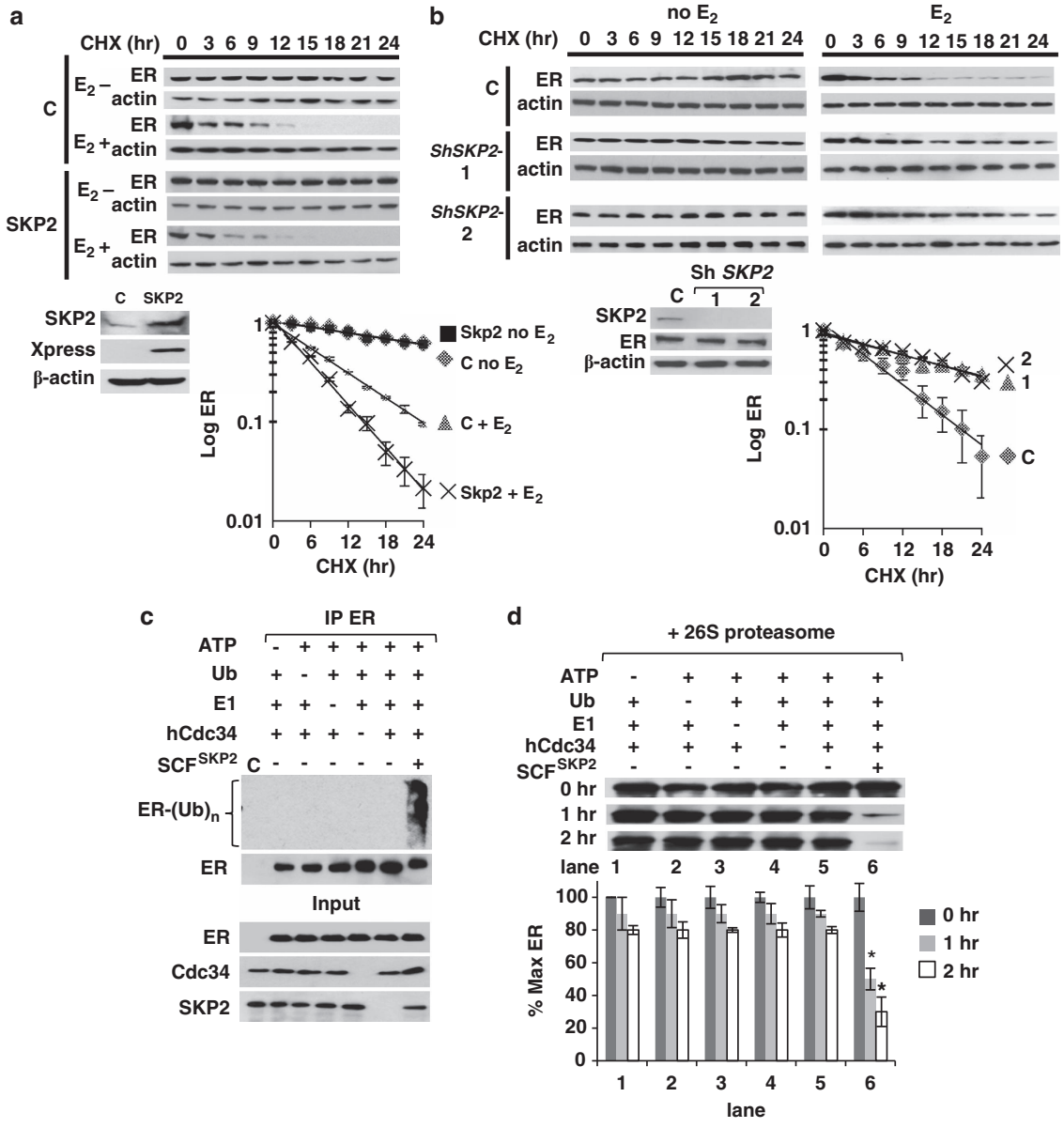

Figure 2. SKP2 levels alter ERα stability and SCF^{SKP2} stimulates ERα ubiquitylation and proteolysis in cells and *in vitro*. (**a**) MCF-7 was stably transfected with empty vector (**c**) or SKP2 then E2 deprived (E2-) or treated with E2 for 2 h (E2 +) followed by addition of CHX and ERα assayed by western blot at intervals shown (top). Western blot shows SKP2 with anti-SKP2 or anti-Xpress-tag antibody (middle). ERα decay was assayed by densitometry, and mean data from three independent experiments performed in triplicate were plotted as semi-log values relative to ERα steady-state level at the time CHX addition. (bottom). Two hours after E2 addition, ERα has a $t_{1/2}$ of 6 h in controls, and a $t_{1/2}$ of 4.5 h in MCF-SKP2 (mean ± s.e.m.). (**b**) MCF-7 cells were infected with scramble shRNA (**c**) or one of two different SKP2 shRNA lentiviri (shSKP2-1, 2). Stable lines were E2 deprived then treated with vehicle (E2-) or E2 for 2 h then CHX added and ERα assayed by western blot at intervals shown (top). ERα decay was assayed by densitometry, and data from three independent experiments performed in triplicate plotted as semi-log values as in (**a**). Western blot shows SKP2 knockdown (lower panel). β-Actin serves as loading control. ERα $t_{1/2}$ is 6 h in E2-stimulated controls, and $t_{1/2} = 11$ h with stable SKP2 knockdown (mean ± s.e.m.). (**c**) For ERα ubiquitylation *in vitro*, ERα, ATP, ubiquitin, E1 and His-hCdc34 were incubated with SCF^{SKP2} per Materials and methods section and ERα precipitates blotted with anti-ubiquitin. (**d**) *In vitro* degradation assay was as in (**c**) with the addition of 26S proteasome complex for the indicated times followed by western blot for ERα. Data from three independent experiments performed in triplicate were plotted as percent of remaining ERα protein level (± s.e.m.) relative to ERα protein level at the starting time. In lane 6, ERα levels were significantly lower at 1 and 2 h compared with $T = 0$ controls two-tailed Student's *t*-test (★ and * signify $P < 0.05$).

ERαS341A, which was less than that of WT-ERα (Supplementary Figure S2C). ERαS294A ubiquitylation was similar to that of WT-ERα both with and without prior cyclin E-CDK2 treatment. SCF^{SKP2}-mediated *in vitro* proteolysis of WT-ERα was stimulated by cyclin E-CDK2 pre-treatment, whereas ERαS341A was unaffected by cyclin E-CDK2 and resistant to proteolysis (Supplementary Figure S2C). Thus, although CDK2-mediated *in vitro* phosphorylation of ERα is attenuated by mutations affecting both S294 and S341, the S341 site constitutes the 'phosphodegron' for estrogen-driven ERα and SKP2 association.

SKP2-ERα complex formation is biphasic during estrogen-stimulated cell cycle re-entry

Estrogen deprivation of MCF-7 induces quiescence, and E2 repletion rapidly activates both cell cycle re-entry[4] and ERα

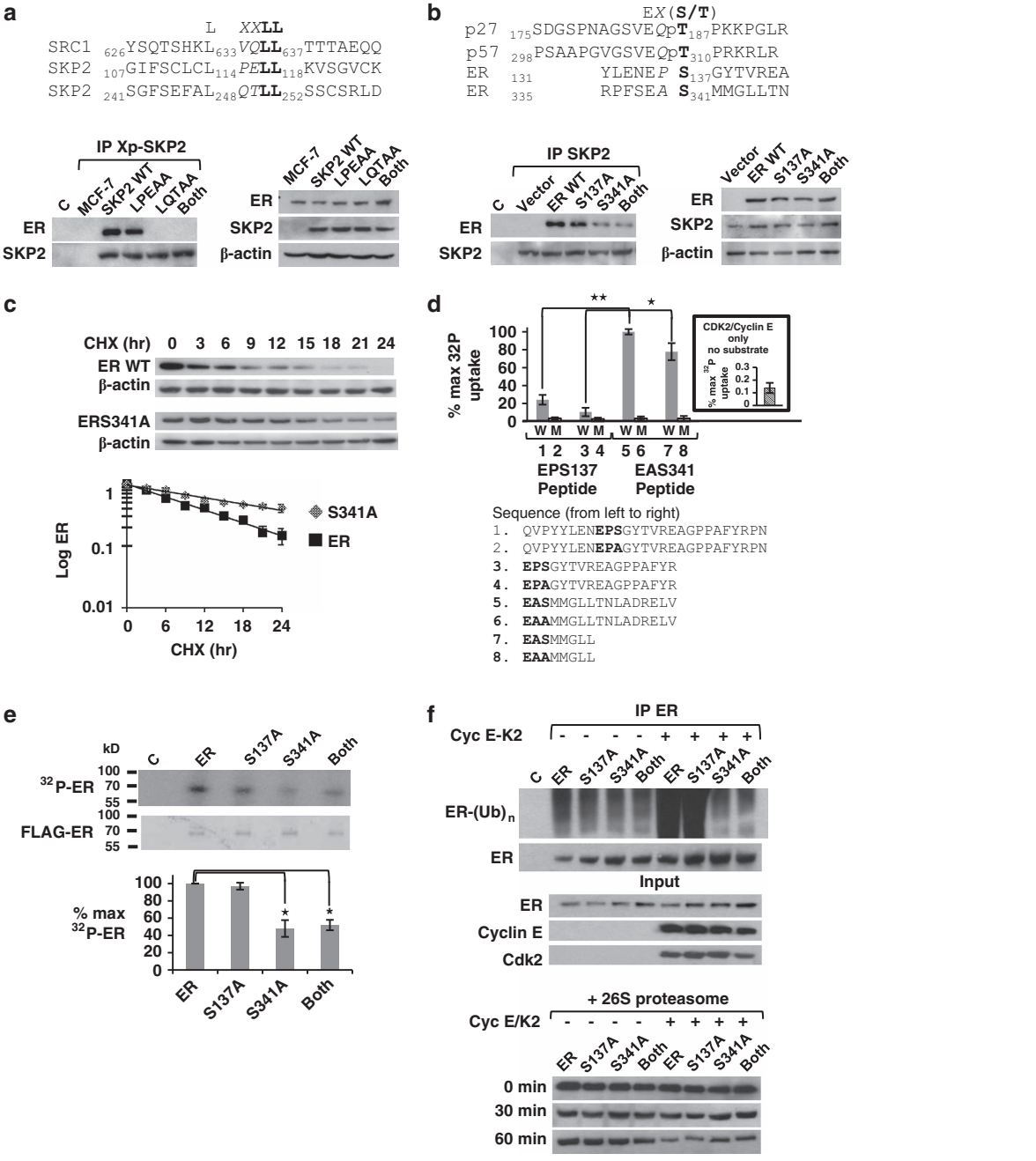

Figure 3. SKP2 $L_{248}QTLL_{252}$ motif is critical for ERα-SKP2 binding, and ERα S341 phosphorylation by cyclin E-CDK2 primes ERα binding and degradation by SCF^{SKP2} *in vitro*. (**a**) Sequence alignment of L*XX*LL motifs from SRC1 and SKP2 on top. Bold type indicates leucines mutated to alanine in SKP2 mutants. MCF-7 stably transfected with vector, WT Xpress-SKP2 or Xpress-SKP2 mutants (SKP2 $L_{114}PEAA_{118}$, SKP2 $L_{248}QTAA_{252}$ or SKP2-2X L*XX*AA) were assayed for Xpess-SKP2-bound ERα by IP-blot. Antibody without lysate served as control. (**b**) Sequence alignment of EX*T*/S motifs from p27, p21 and ERα on top. Bold type indicates serines mutated to alanine in ERα mutants. ERα-negative MDA-MB-231 cells stably transfected with vector only (vector), WT-ERα (ERα WT) or ERα mutants (S137A, S341A or S137A-S341A) were assayed for SKP2-bound ER. Antibody without lysate served as control. (**c**) Stably transfected MDA-MB-231 ERα WT and ERα S341A were E2 deprived, treated with vehicle (E2-) or estradiol (E2 +) for 2 h then CHX added and ERα assayed by western blot at intervals shown. β-Actin serves as loading control. Densitometry and linear regression of data from three independent experiments performed in triplicate shows ERα WT has a $t_{1/2}$ of 6 h, and ERα S341A has a $t_{1/2}$ of 12 h (mean ± s.e.m.). (**d**) ERα WT and mutant peptides were reacted with cyclin E/CDK2 kinase *in vitro*. Radioactivity in ERα peptides was quantitated by liquid scintillation; data were normalized to highest read, and graphed as means ± s.e.m. from triplicate assays. Insert (right) shows control radioactivity recovered on the filter when substrate was omitted from the reaction. Significance was determined by two-tailed Student's *t*-test by comparing S341 containing peptides with S137 containing peptides (comparison were done for both long forms and short forms) ($^{\star}P<0.05$ or $^{\star\star}P<0.01$). (**e**) Recombinant FLAG-ERα WT, ERα S137A, ERα S341A and ERα S137A-S341A were used as substrate for *in vitro* kinase. Coomassie staining shows equal input of each purified protein. Control (c) reactions contained all reagents except substrate. Activity of recombinant ERα proteins was quantitated by liquid scintillation counting; data were normalized to highest read, and graphed as means ± s.e.m. from triplicate assays. Significance was determined using a two-tailed Student's *t*-test by comparing radioactivity in WT recombinant ERα protein with that in ERα mutants (* or $^{*}P<0.05$). (**f**) Recombinant ERα WT, ERα S137A, ERα S341A and ERα S137A-S341A were used as substrate for *in vitro* ubiquitylation and degradation assays with or without cyclin E/CDK2 pre-treatment.

proteolysis.[7,25] MCF-7 cells were synchronized in G0/G1 by 48 h E2 deprivation. Cell cycle profiles after E2 addition showed early S-phase entry by 12 h, with peak S-phase at 21 h (Figures 4a and e). Cellular SKP2 levels were minimal in G0/early G1, rising at the G1/S transition (Figure 4a). Cyclin E and CDK2 protein levels were unchanged during G1-to-S phase, but T160-phosphorylated CDK2 increased[26] (Figure 4a) with cyclin E-CDK2 activation. Cyclin E-CDK2 activity increased by 8–12 h and peaked by 16 h, before peak S phase (Figures 4b and e).

Despite the decline in ERα, its co-precipitation with CDK2 increased during G1 (Figures 4c and e). Notably, although ERα levels fell and SKP2 levels increased during G1 to S phase, ERα-SKP2 binding increased in late G1, peaking after cyclin E-CDK2 activation (Figures 4b, d and e and Supplementary Figure S3B). The kinetics of these events, graphed in Figure 4e, support a model in which activated cyclin E-CDK2 binds and phosphorylates ERα to prime its recognition by SKP2.

Recent work suggests that ERα phosphorylation at S294 by MAPK promotes SKP2 binding.[24] Estrogen rapidly activates MAPK within 5 min, with inactivation by 6 h (Figure 4f and Supplementary Figure S3A). In early G1, SKP2 $t_{1/2}$ and levels are low[27] (Figure 4b). Despite low SKP2 levels, ERα-SKP2 complexes were detected 1 h after E2 (Figure 1c), considerably before cyclin E-CDK2 activation. Comparison of early and late time points revealed two phases of ERα-SKP2 binding (Figure 4f and Supplementary Figure S3B). Complexes were absent in estrogen-starved cells, but low ERα-SKP2 complex levels were detected within 5 min after E2 stimulation, co-incident with rapid MAPK activation, remained stable between 15 min to 6 h, then increased dramatically after cyclin E-CDK2 activation in late G1. When SKP2 levels increased, ER levels were significantly decreased, thus relative protein levels did not favor binding in late G1.

We next compared effects of CDK2 (Roscovitine) and MEK (U0126) inhibitors. Both blocked E2-stimulated G1- to-S-phase progression (Figure 4h). U0126 abolished both the rapid E2-driven MAPK activation and early ERα-SKP2 complex assembly, whereas Roscovitine did not affect either. However, the CDK2 inhibition by Roscovitine abolished the dramatic late G1 rise in ERα-SKP2 complexes (Supplementary Figure S3B).

MEK inhibition prevented cyclin E-CDK2 activation, arrested cell cycle progression and both early and late phases of ERα-SKP2 assembly were lost (IP-blots and quantitation shown in Figures 4f and g with greater detail in Supplementary Figure S3). These data suggest that both MAPK and CDK2 may promote ERα-SKP2 binding, with the former having an early role and CDK2 driving late assembly.

In E2-deprived cells, ERα is stable.[6] E2 addition rapidly (within minutes) stimulates ERα proteolysis.[25,28] As cyclin E-CDK2 activation and SKP2 rise in late G1, SKP2 would affect a later phase of E2-activated ERα degradation. This model would predict that ERα proteolysis kinetics may differ between early and late G1. CHX chase experiments started at 3 and 12 h after estradiol addition, respectively, showed ERα $t_{1/2}$ of 6 h in early G1 and a $t_{1/2}$ of 5 h in late G1/S (Figure 4i). This bimodal pattern with different ERα half-lives early and late after estrogen stimulation, suggests early and late mechanisms govern E2-activated receptor proteolysis.

SCF^{SKP2} regulates ERα target gene expression for G1/S transition and S-phase progression

If SKP2 acts as both ubiquitin ligase and as coactivator for ERα, SKP2–ERα-driven gene targets would be induced late in G1, after cyclin E-CDK2 activation. To identify putative SKP2-coactivated ERα target genes whose expression increases in late G1/S, MCF-7 gene expression profiles were compared before and early (at 3, 6 h) or late (12 h) after E2 stimulation. Total RNA from triplicate samples was extracted, labeled and hybridized to Agilent whole-genome arrays representing >41 000 transcripts (Figure 5a).

Differential expression was assessed as the average ratio between two treatment conditions, with >2-fold change with a false discovery rate <0.05. Late estrogen-activated genes were selected using a cutoff of 1.5-fold increase between 6 and 12 h. Twenty-two genes were upregulated in late G1 by this criterion (Figure 5b and Supplementary Table S1). Many of these are involved in the G1/S transition or mitosis (*E2F-1, FBXO5/EMI1*), and in DNA replication (*BLM, CDC6, RFA*). Over 80% of the genes we found increased by >1.5-fold between 6 and 12 h after E2 addition were also upregulated by E2 in three other publically available array databases.[29–31]

Of these late E2-activated genes, several contained ERα-binding AP-1/Sp-1 sites within 10 kb of their promoter start sites and were predicted by the Hormone Receptor Target Database[32] to be ERα targets (Supplementary Table S2). Several also bear partial ERE consensus motifs in their promoters (Figure 5b). Q-PCR confirmed late upregulation for two of these, *E2F-1* and *BLM* (Figure 5d). E2 stimulated a modest early *E2F-1* induction within 3–6 h in both MCF-7 and ZR-75-1, but *E2F-1* mRNA levels then rose significantly by 12 h (Figures 5d and f).

In both MCF-7 and ZR-75-1, *SKP2* knockdown delayed and attenuated peak S-phase entry (Figures 5c and e) and decreased the late induction of *E2F-1* and/or *BLM* (Figures 5d and f). Notably, the early *E2F-1* induction by estrogen was not affected by *SKP2* knockdown, but its late G1 upregulation was. Not all E2-driven genes expressed late were SKP2 regulated: the late E2-activated *RAB31* induction was not affected by SKP2 knockdown. Canonical ERα target genes, such as *pS2* and *GREB1* were rapidly activated by E2 stimulation, but were not affected by SKP2 knockdown (Supplementary Figure S4).

WT-SKP2 overexpressing MCF-7 showed a notable increase in late E2-mediated *E2F-1* and *BLM* induction at 12 and 24 h after E2 addition (Figures 5g and h); this was not seen in cells over-expressing the C-terminal L*XX*LL mutant, SKP2-L_{248}*QT*LL_{252}. SKP2-L_{114}*PE*AA_{118} did not differ from SKP2 WT in its effects on *E2F-1* and *BLM* activation.

SCF^{SKP2} binds late ERα target gene promoters

E2F-1 and *BLM* are known to be upregulated by ERα/Sp-1 or ERα/AP-1 binding.[33,34] To further investigate whether SKP2 coactivates ERα at these target genes, SKP2 and ERα binding to their Sp-1/AP-1 promoter elements was assayed by chromatin immunoprecipitation (ChIP). ERα occupied both *E2F-1* (Figures 6a and b) and *BLM* (Figure 6c) promoters late after E2 stimulation. Binding increased between 12 and 18 h and was inhibited by tamoxifen. In both MCF-7 and ZR-75-1, ChIP/re-IP showed late E2-stimulated *E2F-1* promoter co-occupancy by ERα, SKP2, SRC-3 and RNA polymerase II that was blocked by tamoxifen pre-treatment (Figures 6a and b). Similar findings were observed for *BLM* (Figure 6c).

Finally, Xpress-WT-SKP2, but not the L*XX*LL mutant SKP2-L_{248}*QT*AA_{252}, showed a considerable increase in binding at *E2F-1* and *BLM* promoters at 12 and 24 h after estrogen stimulation (Figure 6d). Binding of the more proximal L*XX*LL mutant SKP2-L_{114}*PE*AA_{118} to the respective *E2F-1* and *BLM* promoter Sp-1/AP-1 elements did not differ significantly from WT-SKP2. Taken together, these data suggest that *E2F-1* and *BLM* are part of a subset of late-activated ERα target genes coactivated by SKP2.

DISCUSSION

As for many TFs,[14] ERα activation by ligand rapidly stimulates both its transcriptional competence and its ubiquitin-dependent degradation.[35] Although certain E3 ligases serve as ERα coactivators,[25] how and at what promoters transcription-coupled ERα proteolysis occurs is not fully known. Present data support a model in which SCF^{SKP2} serves as a dual E3 ligase and ERα

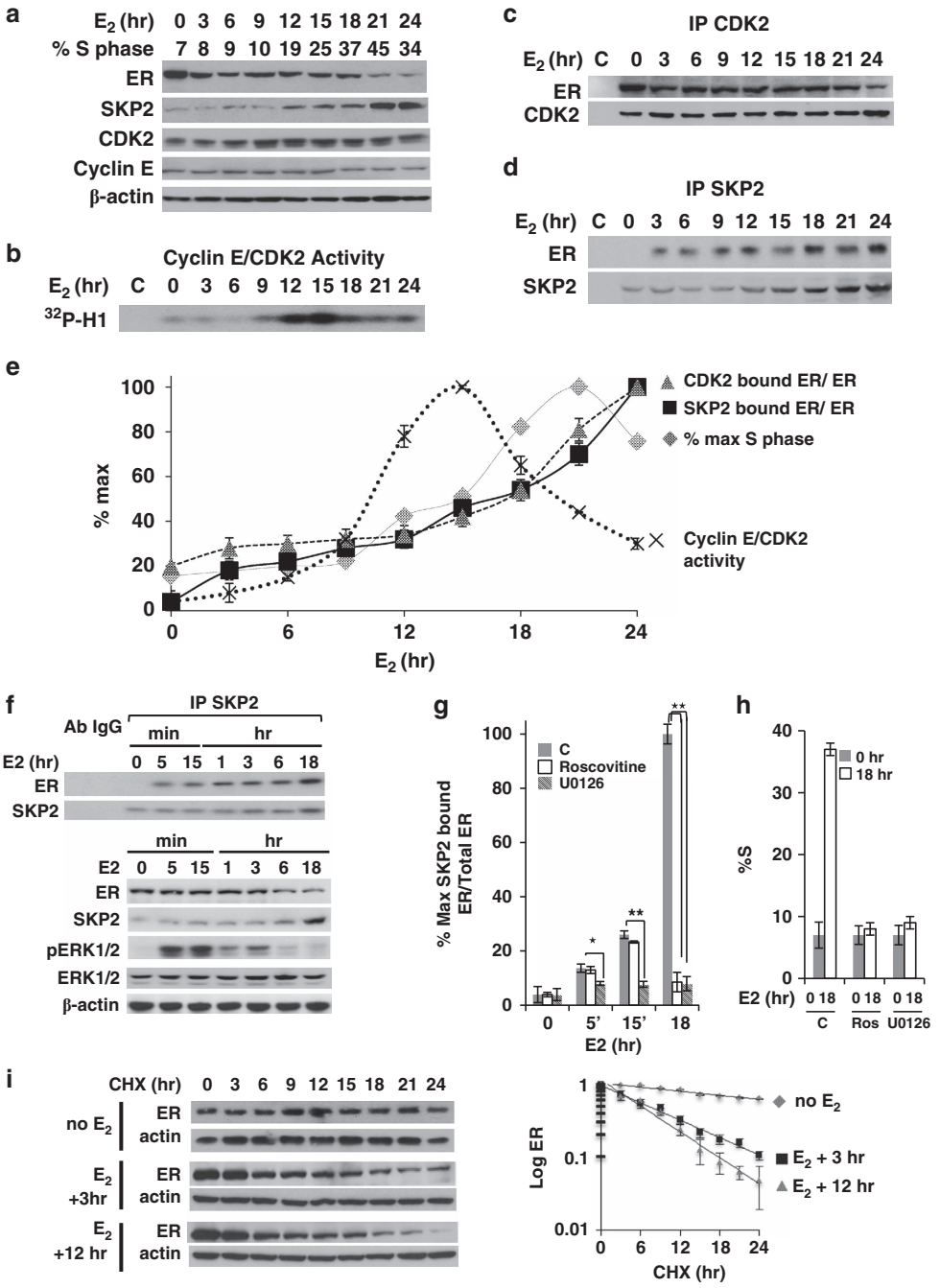

Figure 4. Quantitation of cyclin E-CDK2 activity, CDK2-bound and SKP2-bound ERα and ERα $t_{1/2}$ during G1-to-S-phase progression. (**a**–**e**) E2-deprived MCF-7 were treated with 10^{-8} M E2 and recovered at intervals shown. (**a**) Flow cytometry showed cell cycle re-entry (% S phase), and western blots for ERα, cyclin E, SKP2 and CDK2 are shown. β-Actin serves as loading control. (**b**) For cyclin E-CDK2 kinase activity, cyclin E was precipitated and reacted with histone H1 and radioactivity in substrate (^{32}P-H1) shown by autoradiography. Activity was quantitated by phospho-imager and graphed as mean % max activity ± s.e.m. from >3 assays in (**e**) below. (**c**, **d**) CDK2-bound ERα (**c**) and SKP2-bound ERα (**d**) assayed at intervals after E2 addition. (**e**) Quantitation of data from **a**–**c** above. The experiments were repeated at least three times. Data were normalized to highest read, and graphed as mean % max ± s.e.m. from >3 assays. (**f**) E2-deprived MCF-7 were treated with E2 for intervals shown, lysed and assayed for SKP2-bound ERα. Lanes 1 and 2 are antibody control and normal immunoglobulin G control for IP. Lower panel shows input on western blot. (**g**) SKP2-bound ERα was assayed at intervals after E2 addition ± pre-treatment with CDK2 inhibitor Roscovitine or MEK inhibitor U0126, and graphed as mean % max ± s.e.m. from >3 assays (see also Supplementary Figure S2). Significance was determined using a two-tailed Student's *t*-test by comparing control with that from drug pre-treatment (★ $P<0.05$ or ** $P<0.01$). (**h**) Flow cytometry (% S phase) showed cell cycle re-entry at 18 h in control but not in drug treated cells. (**i**) E2-deprived MCF-7 were treated with E2 for 3 or 12 h before adding CHX and harvested at various time points for ERα western blot. β-Actin serves as loading control. After 3 h of E2, ERα has a $t_{1/2}$ of 6 h, whereas after 12 h of E2, ERα has a $t_{1/2}$ of 5 h.

coactivator to link liganded-ERα activation with late induction of genes that mediate G1-to-S-phase and later cell cycle events. We show the F-box protein, SKP2, bears two signature L*XX*LL coactivator motifs, and binds ERα via the distal site at aa 248–252. *SKP2* overexpression and knockdown modulate ligand-activated ERα degradation in cells, and priming phosphorylation

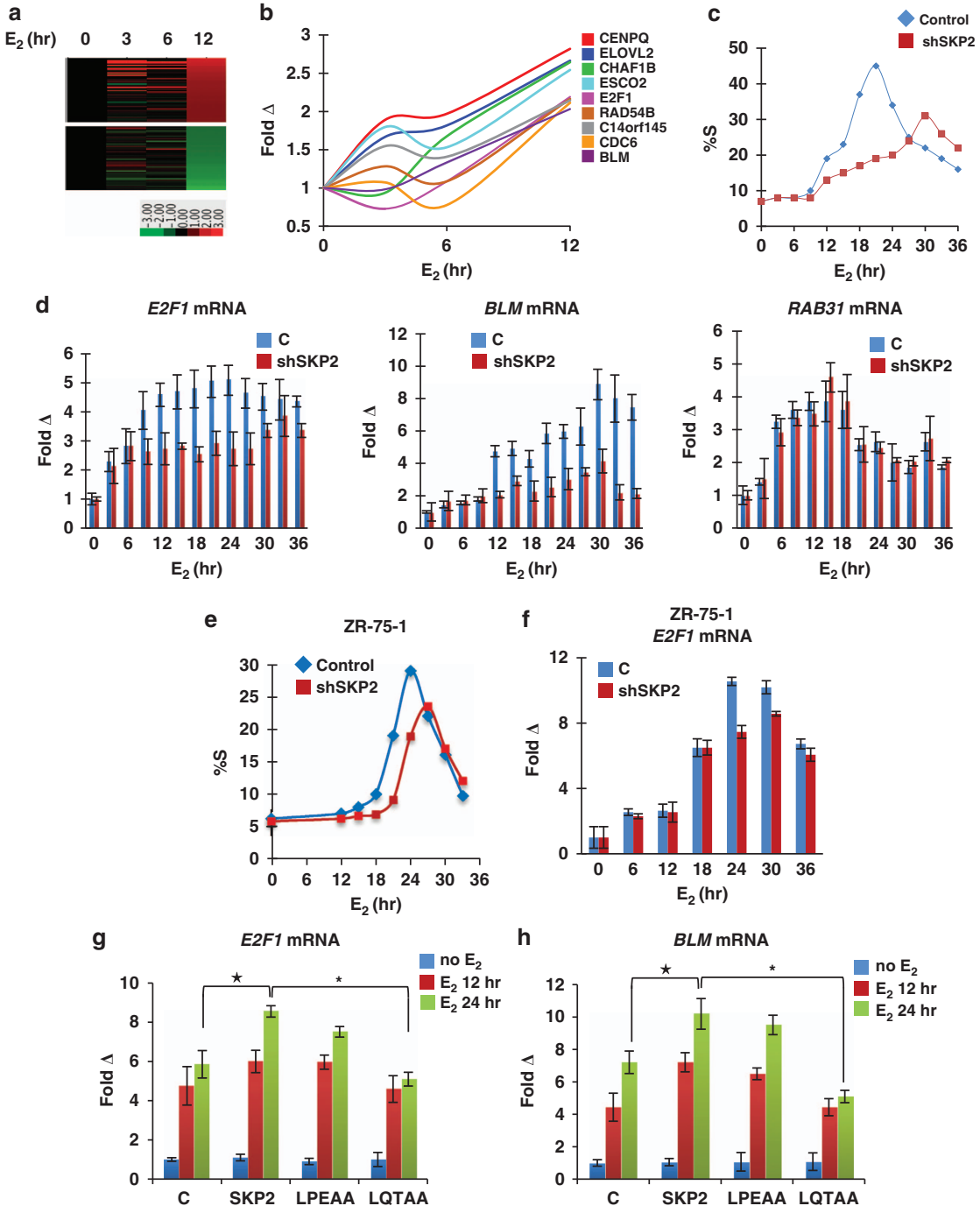

Figure 5. *E2F-1*, *BLM* are part of a subset of late ERα-activated genes regulated by SCF^{SKP2}. (**a**) Heatmap of E2-stimulated gene expression. Clustering of genes expressed in E2-deprived MCF-7 cells treated with 10^{-8} M E2 for 3, 6, 12 h compared with untreated (see also Supplementary Tables S1 and S2). (**b**) Plot of eight ERE-bearing genes whose expression rises >1.5X between 6 and 12 h after E2 stimulation (see also Supplementary Tables S1 and 2). (**c**, **d**) E2-deprived parental MCF-7 or MCF-7 SKP2 knockdown (MCF-7/shSKP2) cells were treated with E2 and assayed at intervals for: (**c**) Cell cycle profile by flow cytometry, graphed as mean % S-phase cells/time in MCF-7 ± ShSKP2. (**d**) *E2F-1*, *BLM* and *RAB31* transcripts quantitated by reverse-transcription q-PCR. *GAPDH* was used as an internal control. Data from three biologic experiments are plotted as fold induction over control (mean ± s.e.m.). (**e**, **f**) Cell cycle profiles (**e**) and q-PCR for *E2F-1* transcript quantitation (**f**) were carried out as (**c**, **d**) in a second ERα-positive cell line ZR-75-1 and derivative ZR-75-1/shSKP2. (**g**, **h**) MCF-7 transfected with either WT-SKP2, SKP2-$L_{114}PEAA_{118}$ or SKP2-$L_{248}QTAA_{252}$ were E2 deprived and recovered 12 and 24 h after E2 addition for q-PCR of *E2F-1* (**e**) and *BLM* (**f**). Significant differences were determined using a two-tailed Student's *t*-test comparing target gene mRNA levels with WT SKP2 overexpression with that of control cells or cells overexpressing SKP2 mutant $L_{248}QTLL_{252}$ (★ and $*P<0.05$).

of ERα by cyclin E-CDK2 increases SKP2-mediated ERα ubiquitylation and proteolysis *in vitro*. ERα-SKP2 complexes are absent in E2-deprived cells and estrogen stimulates early rapid MAPK activation and low level ERα-SKP2 binding followed by a more dramatic rise in ERα-SKP2 binding after cyclin E-CDK2 activation in late G1. Loss of potential to phosphorylate ERα at a SKP2-binding E*X*S motif surrounding ERαSer341 abrogates both ERα-SKP2 binding and the priming effect of cyclin E-CDK2 on SKP2-mediated ERα proteolysis

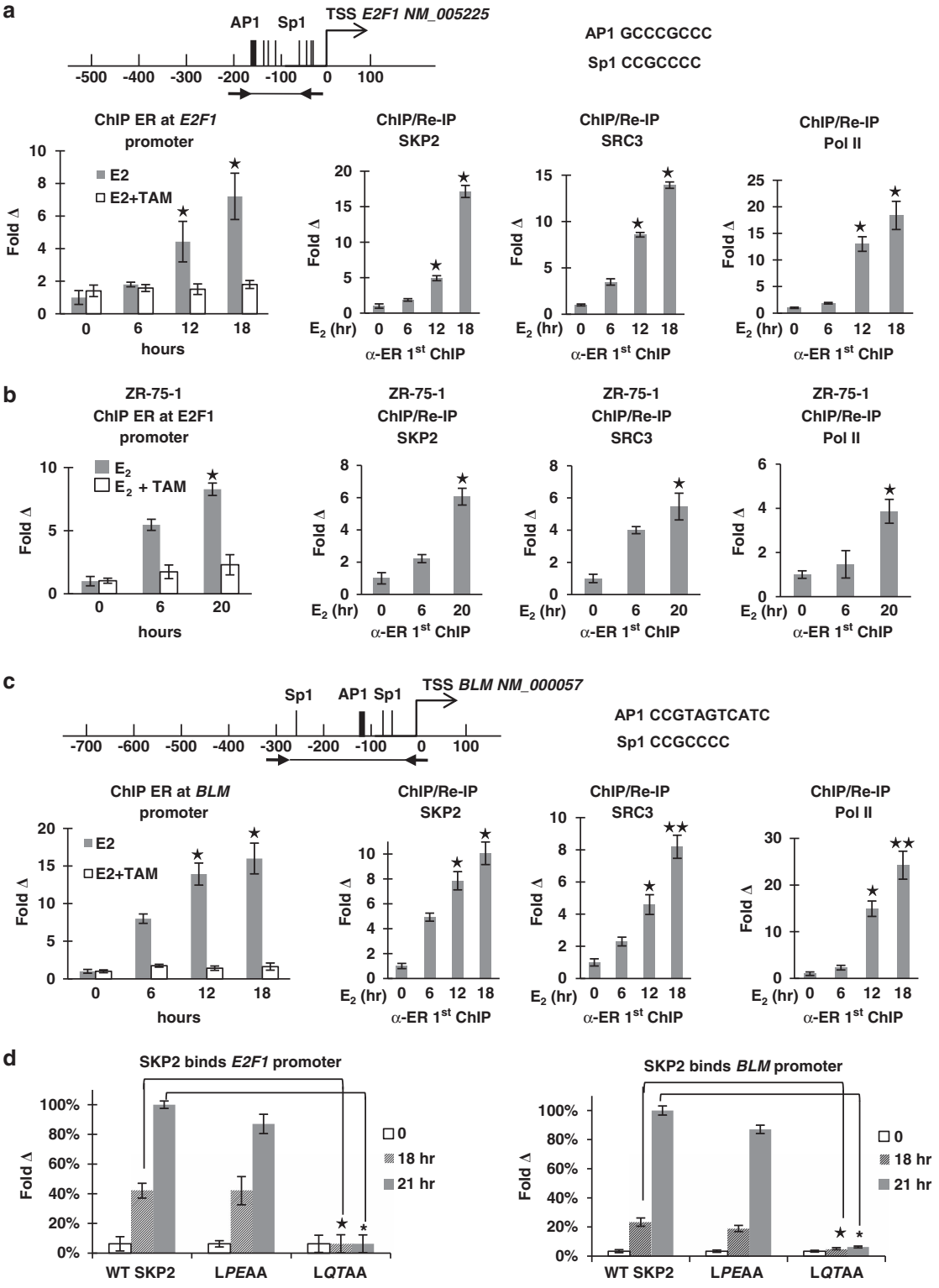

Figure 6. SKP2 directly regulates late ERα-activated genes *E2F-1* and *BLM*. MCF-7 or ZR-75-1 cells were harvested after 48-h E2 deprivation at time = 0 h or after 10 nM E2 for the times indicated. (**a**, **b**) ERα ChIP was performed at the *E2F-1* promoter at indicated times in MCF-7 (**a**) or ZR-75-1 (**b**). ERα ChIP/Re-IP used SKP2, SRC3 or polymerase II (Pol II) Abs for the re-precipitation. The fractions of protein bound to the promoter at different E2 treatment intervals were compared with that of E2 depleted cells and significant differences were determined using a two-tailed Student's *t*-test ($^{\star}P<0.05$). (**c**) ERα ChIP was performed at the *BLM* promoter. ERα ChIP/Re-IP used antibodies to SKP2, SRC3 or Pol II. The fractions of protein bound to the promoter at different E2 treatment intervals were compared with that of E2 depleted cells and significant differences were determined using a two-tailed Student's *t*-test ($^{\star}P<0.05$ and $^{\star\star}P<0.01$). (**d**) MCF-7 cells stably expressing Xpress-tagged WT SKP2, SKP2-L_{114}PEAA_{118} or SKP2-L_{248}QTAA_{252} were harvested after 48 h E2 deprivation at time = 0 h or after 10 nM E2 treatment for 18 or 21 h. ChIP experiments were performed at *E2F-1* or *BLM* promoters using anti-Xpress antibody. Significance was determined using a two-tailed Student's *t*-test by compared the fraction of WT-SKP2 protein binding to gene promoter at 18, 21 h of E2 with that of cells expressing SKP2 L_{248}*QT*LL_{252} (*, $^{*}P<0.05$).

in vitro. Expression profiling identified estrogen-induced genes that rise late, at the G1-to-S transition. Of these, *E2F-1* and *BLM*, are identified as SKP2-dependent ERα targets, whose transactivation involves estrogen-stimulated ERα, SKP2, SRC-3 and polymerase II promoter occupancy. As E2F-1 transactivates both *cyclin E* and *SKP2*, present findings support a feed forward mechanism whereby late SKP2-dependent ERα-driven gene induction would feed forward to drive S-phase entry.

Several E3 ligases have been proposed to have roles in ERα degradation including MDM2,[17] E6-AP,[16] BRCA1-BARD1[36] and cullin-based RING finger type E3 ligases, CUL4B-AhR,[37] CUL5[38] and CUL7.[24] Although multiple cullin-based RING ligases may regulate ERα in a cell-type-dependent manner, potential effects of these E3s on ERα transactivation function remained largely unknown. Our preliminary observation that Cul1-bound ERα and *DN-Cul1* attenuated E2-activated ERα loss, stimulated further investigation of SCFs as ERα ubiquitin ligases. SCF^{SKP2} regulates degradation of CDK inhibitors,[21,39] cyclin E[40] and factors involved in DNA replication (Cdt1),[41] and repair (BRCA2 and NBS1).[42,43] Present work suggests a novel SKP2 function linking estrogenic ERα activation to cell cycle progression with periodic, estrogen-driven interaction between cellular SCF^{SKP2} and ERα as substrate in late G1. This contrasts with a recent report of estrogen-independent SKP2-mediated ERα proteolysis that involves a Cul7 complex.[24]

Cell cycle progression requires successive cyclin-CDK activation. CDK2 regulates the G1/S transition. Although CDK1 can compensate genetic *CDK2* loss in $CDK2^{-/-}$ mice,[44] micro-injected antibodies to cyclin E or CDK2 arrest somatic cells in G1.[45,46] In late G1 and S phases, cyclin E-CDK2 phosphorylates nuclear substrates, including the retinoblastoma protein, histone H1, CDC6 and other proteins involved in DNA replication.[19] Present findings suggest a model in which ERα phosphorylation by cyclin E-CDK2 drives activation of late ERα target genes to promote G1/S progression and beyond. Although a majority of CDK2 substrates have a S/T-P central motif, the putative CDK2 site at S341 in ERα lacks this motif. Notably, only half of identified substrates have this full consensus and at least 10% of CDK2 substrates have been shown not to be proline directed.[47] Of the non-proline directed CDK2 substrates, about half contain RXLΦ or K/RXLXΦ motifs that promote CDK2 substrate binding. Notably, the putative CDK2 phosphorylation site at ERαS341 is followed by two R*X*LΦ motifs ($R_{352}ELV_{355}$ and $K_{401}LLF_{404}$) that would promote CDK2-substrate binding.[48]

Liganded ERα promotes G1 transit by cross talk between liganded ERα and Ras-Raf-MAPK[1] and by *cyclin D1* induction in early G1, to activate CDK4 and CDK6, followed by cyclin E-CDK2 activation. These CDKs phosphorylate and inactivate the retinoblastoma protein, releasing activated E2F-1.[49] E2F-1 in turn transactivates *cyclin E* and *cyclin A* genes.[49] Thus, cyclin E-CDK2 creates a positive feedback loop through E2F-1, to induce further *cyclin E*. E2F-1 also drives *SKP2* expression,[50] which further activates cyclin E-CDK2 via SKP2-mediated degradation of the CDK inhibitor, p27.[51] Cyclin E-CDK2 also phosphorylates p27 to prime its degradation by SCF^{SKP2}.[51] As a check on this process, SKP2 autoregulates by mediating both E2F-1[52] and cyclin E degradation.[40,53]

Present data reveal a novel mechanism of *E2F-1* induction in ERα-positive breast cancer cells (Figure 7). In hormone-sensitive tissues, liganded ERα translocates to the nucleus where cyclin E-CDK2 phosphorylates ERαS341 to prime its binding to SKP2, and transactivation of *E2F-1*. Increased *E2F-1* expression, together with E2F-1 activation via cyclin D- and cyclin E-CDKs, would feed forward to further induce *SKP2* , *cyclin E*, *cyclin A* and other E2F-1 target genes that drive S and G2/M progression.[49]

Although we observed cyclin E-CDK2-mediated ERαS341 phosphorylation primes SKP2 binding, ERα ubiquitylation and degradation *in vitro*, Bhatt *et al.*[24] suggested that p38 MAPK-driven ERα phosphorylation at S294 mediates SKP2 binding and receptor

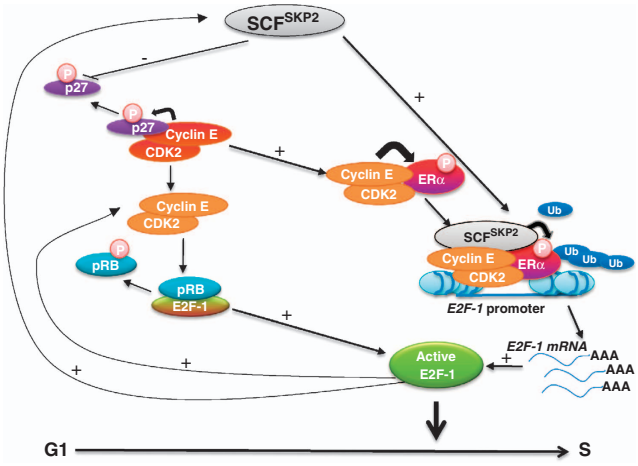

Figure 7. Model of E2F-1 activation via classic retinoblastoma protein (pRb) pathway or via novel ERα/SCF^{SKP2} pathway. G1 progression is stimulated by SKP2-mediated p27 degradation, which frees cyclin E-CDK2 to phosphorylate pRB, releasing activated E2F-1. Activated cyclin E-CDK2 phosphorylates ERα, priming ERαSCF^{SKP2} binding and *E2F1* transactivation in late G1. E2F1 induction and activation feed forward to induce further cyclin E, A and SKP2, driving late G1 and S-phase entry.

proteolysis and showed ERαS294A was stable and unaffected by SKP2 overexpression. Present data allow a model in which ERα phosphorylation at S294, which is observed within 30 min after E2 addition,[54,55] co-incident with rapid transient MAPK activation, may prime low level early ERα-SKP2 binding. Both pMAPK and early ERα-SKP2 binding were abolished by U0126, but were unaffected by the CDK2 inhibitor, Roscovitine, which did not inhibit early MAPK activation. However, since both U0126 and Roscovitine prevent G1-to-S progression, they both prevent the late, more dramatic CDK2-dependent rise in ERα-SKP2 following cyclin E-CDK2 activation. Bhatt *et al.*[24] showed DN-MAPK, and inhibitors of p38 MAPK and CDK2 (all of which block G1-to-S transit), all impaired the increase in ERα ubiquitylation caused by SKP2 overexpression, compatible with our data. Our observation that ERαS294A was not resistant to SCF^{SKP2}-mediated ubiquitylation while ERαS341A was, together with the observed priming effect of CDK2 on ERα-ubiquitylation *in vitro*, allow one to speculate that early S294 phosphorylation may initiate complex formation, which is then more dramatically catalyzed by sustained cyclin E-CDK2 action during late G1, to drive SKP2 coactivation of novel late ERα target genes and receptor turnover.

Cyclin E-CDK2 mediated phosphorylation of ERα leads to SKP2-dependent transcription of *E2F-1, BLM* and potentially other late S-phase and G2/M regulators. This is reminiscent of links between G1/S CLN-CDC28 and S-phase promoting CLB-CDC28 complexes in *S. cerevisiae*. In yeast, the G1 CDK complex, CLN-CDC28, phosphorylates TFs like PHO4 that activate CLB-CDC28 to drive S-phase gene expression and G2/M progression.[19] Similarly, cyclin E-CDK2-mediated activation of ERα-SKP2 would drive *E2F-1* transactivation to promote S-phase entry and G2/M CDK activation. Our expression profiling identified many late E2-responsive genes upregulated between 6 and 12 h, which encode TFs governing S phase (E2F-1 and E2F-8), DNA replication (CDC6, BLM and RFC3) or mitosis (FBXO5/EMI1 and CENPQ). Notably, several contain both ERE half-sites and Sp-1/AP-1 motifs in their promoters. Of these, the Sp-1/AP-1 sites on *E2F-1* and *BLM* promoters were shown to be hormone regulated, with late E2-driven promoter occupancy by ERα-SKP2.

Typically, E2-activated genes induced early within 1–3 h by liganded ERα, such as *c-Myc* , involve ERα interaction at AP-1/Sp-1 sites;[56] whereas ERE-containing genes are activated by 4–6 h.[57]

Although most genes induced late after E2 may be upregulated secondarily by other TFs, *E2F-1* and *BLM* appear to be induced by ERα. Whether very late-activated ERα targets, upregulated more than 12 h after E2, are regulated more often via an Sp-1/AP-1 mode, as observed for *E2F-1* and *BLM*, or involve ERE sites warrants further investigation. The present work identifies these late-activated genes as *bona fide* ERα targets and describes a novel mechanism of their periodic coactivation via SKP2. Whether this or similar mechanisms drive late activation of other hormone receptor targets will need further evaluation.

Deregulation of G1-S progression is a hallmark of cancer. Aberrant CDK2 activation is frequent in human cancer.[58] That CDK2 inhibition blocks proliferation of cultured cancer cells,[45,46] suggests that they may be 'oncogene-addicted' to CDK2. In cancers, CDK2 activation results from increased cyclin E, loss of p27 and/or SKP2 overexpression.[51] Notably, several primary breast cancer studies found a strong association between the ERα-negative status and reduced CDK2 inhibitor p27 protein ($P<0.0001$).[51] A similar relationship was also observed for elevated SKP2 and ERα-negative status.[21,24] Present data suggest that in ERα-positive cancers, deregulated cyclin E-CDK2 would increase estrogen sensitivity by promoting ERα-SKP2 interaction, and the expression of E2F-1 and other ERα target genes to augment mitogenic effects of estrogen.

MATERIALS AND METHODS

Cell culture and cell cycle analysis

MCF-7, MDA-MB-231 and HEK 293T and derivatives were cultured as in Chu *et al*.[59] MCF-7 and shSKP2-MCF-7 cells were E2-depleted in improved minimum essential medium (IMEM) media with 5% charcoal-stripped fetal bovine serum for 48 h. Cell cycle entry was stimulated by E2 (10^{-8} M). Cells were BrdU pulse labeled at intervals and cell cycle analyzed by flow cytometry as in Chu *et al*.[59] ZR-75-1 cells were cultured as in Daly and Darbre.[60] Derivation of SKP2 overexpression and knockdown lines generated from MCF-7 including MCF-7/wtSKP2, MCF-7/SKP2 LPEAA, MCF-7/SKP2 LQTAA, MCF-7/SKP2 2X mut, MCF-7/shSKP2-1, and MCF-7/shSKP2-2 and of MDA-MB-231 variants overexpressing ER-WT or ER mutants is described below. Roscovitine and U0126 were purchased from Cell Signaling Technology (Danvers, MA, USA).

Western blot and CHX chase

Western blot and CHX chase were as described.[28] Antibodies to ER (HC-20, F-10), CDK2 (M2), cyclin E (HE-12, HE111) and ubiquitin (P4D1), were from Santa Cruz (Santa Cruz, CA, USA); to SKP2 (8D9) and Xpress from Invitrogen (Carlsbad, CA, USA); to CUL1, SKP1 and RBX1 from Abcam (Cambridge, MA, USA); to β-actin (C-4) from Millipore (Billerica, MA, USA). The ERα $t_{1/2}$ was determined by CHX chase, with addition of 100 μg CHX considered $t=0$. Cells were lysed at the times indicated and ERα was blotted. For determining the ERα half-life, ERα protein was quantitated by densitometry from 3 experiments using Glyko BandScan software (version 5.0; Glyko, Hayward, CA, USA).

Immunoprecipitation/immunoblotting and immunodepletion

For immunoprecipitations, lysate was precleared with 30 μl of protein A sephorase for 2 h and then incubated with primary antibodies or normal rabbit or mouse immunoglobulin G or antibody without lysate as control (Ab) over night at 4 °C. Complexes were collected on protein A/G Sepharose (Invitrogen) and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by immunoblotting.

Immunoprecipitated cyclin E-CDK2 kinase assays

Cellular cyclin E-Cdk2 complexes were precipitated with anti-cyclin E mAb H111 (Santa Cruz) and kinase activity vs histone H1 assayed and quantitated as in Chu *et al*.[59]

Plasmids, site-directed mutagenesis and transfection

Human SKP2 vector, pcDNA4-Xpress-SKP2 is from H.K.Lin (Houston, TX, USA). Mutagenesis converting lysine 147/148, 251/252 to alanine in pcDNA4-Xpress-SKP2 used QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). MCF-7 cells were transfected with different pcDNA4-Xpress-SKP2 constructs using lipofectamine, and stable lines selected with G418. ER S137A, ER S341A and ER 2X Mut were cloned similarly and stable MDA-MB-231/ER variants generated.

Lentiviral short hairpin RNA (shRNA)-mediated inhibition of SKP2 expression

For lentiviral shRNA production, 293T cells were co-transfected with one of two antisense sequences of SKP2 shRNA (Open Biosystems, Huntsville, AL, USA) or green fluorescent protein control shRNA with packing plasmids (pCMV deltaVPR8.9) and envelope plasmid (pCMV VSV-G) using Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's instruction. After 2 days, virus-containing media were collected, filtered and added to cell monolayers overnight then replaced with fresh growth medium and puromycin 500 ng/ml added. ShRNA sequences used are: SKP2-lentiviral shRNA-1 (5′-GCCTAAGCTAAATCGAGAGAA-3′), SKP2-lentiviral shRNA-2 (5′-CCATTGTCAATACTCTCGCAA-3′) and green fluorescent protein shRNA (5′-GCAAGCTGACCCTGAAGTTC-3′).

Expression and purification of bacculoviral ERα protein and SCF^{SKP2} complex

FLAG-tagged human ERα (or mutant) was purified from insect Sf9 cells infected by recombinant baculoviri harboring the FLAG-tagged ERα-coding sequence derived from pPK-ERα-FLAG (or pPK-ERα-FLAG(S137A, S341A or double mutant) as reported.[61]

SCF^{SKP2} complexes were purified from Sf-9 cells after co-infection with bacculovirus containing each SCF^{SKP2}component (pBac-His-CUL1, pBac-His-SKP1, pBac-RBX1, pBac-GST-SKP2).[21]

In vitro cyclin E-CDK2 kinase assays using recombinant FLAG-ERα or cellular ERα as substrate

Recombinant glutathione *S*-transferase-cyclin E and CDK2 (Millipore) 5 ng was reacted with 1 pmol full-length recombinant FLAG-ERα (WT or S341A or S137A mutants) in 25 μl kinase buffer (0.1 mM ATP, 0.02 μCi/μl [γ-32P]-ATP, 8 mM MOPS/NaOH pH7.0 and 0.2 mM EDTA) at 30 °C for 10 min. Control reactions contained all reagents without substrate. The reactions were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and visualized by autoradiography. Glutathione *S*-transferase -tagged cyclin E (87 kDa) and CDK2 (34 kDa) were readily distinguished from that of ERα protein (66 kDa). To quantitate radioactivity incorporated into ERα, reaction mixtures were dotted onto P81 filtermat, washed 3X with 75 mM phosphoric acid, then once with methanol, and radioactivity in ERα substrate was measured by liquid scintillation. The minimal radioactivity in no substrate controls was subtracted and results were graphed as mean radioactivity in substrate of >3 independent reactions $\pm$ s.e.m.

In vitro kinase assays using cellular ERα as substrate used methods as for recombinant FLAG-ERα proteins. Full-length ERα (WT or mutant ERαS341A, ERαS294A) overexpressed in HEK 293T cells were precipitated with HC-20 antibody from 1 mg cell lysate and used as substrates in cyclin E-CDK2 kinases assays. Control reactions contained all reagents without substrate. Radioactivity in ERα was quantitated and graphed as above. ERα proteins were then subjected to *in vitro* ubiquitylation and degradation assays. For kinase assays, reactions were stopped by adding 2X sodium dodecyl sulfate sample buffer and then resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel electrophoresis, gels dried and visualized by autoradiography.

In vitro kinase assay using ERα peptides

In vitro kinase assays using ERα peptide substrate used methods as for full-length ERα proteins. Reactions were stopped with 5 μl of 3% phosphoric acid, 10 μl aliquot dotted onto P81 filtermat, washed 3X with 75 mM phosphoric acid, once with methanol and radioactivity in ERα peptide substrate measured by liquid scintillation. Control reactions contained all reagents and kinase but no substrate. ERα peptide synthesis used Sigma PEPscreen Custom Peptide Libraries, St Louis, MO, USA. Peptides used are:

(1) $_{127}$QVPYYLENEPSGYTVREAGPPAFYRPN$_{153}$; (2) $_{127}$QVPYYLENEPAGYTVREAGPPAFYRPN$_{153}$; (3) $_{135}$EPSGYTVREAGPPAFYR$_{151}$; (4) $_{135}$EPAGYTVREAGPPAFYR$_{151}$; (5) $_{339}$EASMMGLLTNLADRELV$_{355}$; (6) $_{339}$EAAMMGLLTNLADRELV$_{355}$; (7) $_{339}$EASMMGLL$_{346}$; (8) $_{339}$EAAMMGLL$_{346}$.

In vitro ERα ubiquitylation and proteasomal degradation assays

Ubiquitylation assays were as in Sun *et al.*[25] and used 40 ng each of recombinant ERα (Calbiochem, San Diego, CA, USA), ubiquitin-activating enzyme, his-tagged-Ubc H1, Cdc34, 400 ng SCF^{SKP2} components from bacculovirus, and an energy regenerating system (Boston Biochem, Cambridge, MA, USA) in 7.4 mM HEPES (pH 7.4), 5 mM KCl, and 1.5 mM MgCl2 reacted for 60 min at 30 °C. Reactions were diluted 10-fold in phosphate-buffered saline, and ERα precipitated, complexes were resolved, transferred to nitrocellulose. The membrane was boiled for 10 min. Ubiquitinated ERα was detected by immunoblotting with anti-ubiquitin Ab as in Chu *et al.*[28] ERα degradation was assayed as above, with modifications: 50 nM 26S proteasome fraction (Boston Biochem) was added for 1–2 h at 30 °C before ERα western blot.[28]

Luciferase reporter gene assays and transfections

For reporter gene assays, 2 μg GREB-ERE1-luc and 100 ng pcDNA3-ERα (WT or Mut) or control vectors were transfected and reactions carried out as described.[62]

Gene expression array analyses

RNA was extracted with RNeasy mini kit (Qiagen, Valencia, CA, USA) and processed for Agilent Whole-Genome Oligo microarrays (Agilent, Santa Clara, CA, USA) as per the manufacturer. Arrays were scanned with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA). Raw data were analyzed with GenePix Pro 6.1 (Molecular Devices). Two biologic replicates were used for each treatment. Differential expression and false discovery rate were assessed by linear model and empirical Bayes moderated F statistics.

RNA extraction and gene expression quantification

MCF-7 or MCF-7/ShSKP2 cells were E2-depleted for 2 days before adding 10 nM β-estradiol or 10 μM tamoxifen plus estradiol for 6 or 12 h. Total RNA was isolated using TRIzol (Invitrogen). cDNA synthesis used 1 μg total RNA and iScriptcDNA kit (Bio-Rad, Hercules, CA, USA) and gene expression was quantified by RT–qPCR as described.[62] Primers were: *BLM* 5′-CAGA CTCCGAAGGAAGTTGTATG-3′ and 5′-TTTGGGGTGGTGTAACAAATGAT-3′; *E2F1* 5′-CCAGGAAAAGGTGTGAAATC-3′ and 5′-AAGCGCTTGGTGGTCAGA TT-3′; *RAB31* 5′-GACCCAAGGGCGTGGTCCAC-3′ and 5′-ACATTTCCTGCCCC GCCCCC-3′; and *GAPDH* 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGA TGGTGATGGGATTTC-3′.

Chromatin immunoprecipitation

The ChIP experiments were performed as described.[25] MCF-7, MCF-7/ShSKP2 or Skp2 WT- or mutant expressing MCF-7 cells estrogen deprived, repleted with 10 nM estradiol or vehicle (ethanol) for 45 min, 75 min, 3 h, 6 h or 12 h followed by ChIP assays. The primers for ChIP were 5′-CTGGTACCATCCGGACAAAG-3′ and 5′-ACTTTTACGCGCCAAATCCT-3′; and *BLM* 5′-TGGCAAGTCTCAGCTCTCAA-3′ and 5′-TCCAAAGCCCAATCAG AGTC-3′.

ChIP/Re-IP (sequential ChIP or reChIP)

ChIP/Re-IPs were carried out as described.[62] Bead eluates from the first immunoprecipitation were incubated with 10 mM DTT at 37 °C for 30 min and diluted 1:50 in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl at pH 8.1) followed by a second round of immunoprecipitation.

Statistical analysis

All experiments (in cells and *in vitro* assays) were done at least in triplicate. Data are presented as mean ± s.e.m. as percentage of control or absolute values. The values obtained for WT and mutant proteins for different parameters studied were compared by two-tailed Student's *t*-test. *P*-values <0.05 or 0.01 were designated with one or two asterisks, respectively.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank M Pagano for bacculovirus stocks (CUL1, SKP1, RBX1, SKP2), WL Kraus for pPK-FLAG-ERα plasmid, H-K Lin for pcDNA4-His-Max-Xpress SKP2, and J Sun for technical intellectual input. This work was supported by NIH R01CA123415 (JMS and ZN), and a US DOD Pre-doctoral grant W81XWH-11-1-0097 (WZ).

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)

Appendix 2 Nature Reviews Cancer 2013 reprint

Links between oestrogen receptor activation and proteolysis: relevance to hormone-regulated cancer therapy

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Abstract | Oestrogen receptor-α (ERα) is a master transcription factor that regulates cell proliferation and homeostasis in many tissues. Despite beneficial ERα functions, sustained oestrogenic exposure increases the risk and/or the progression of various cancers, including those of the breast, endometrium and ovary. Oestrogen–ERα interaction can trigger post-translational ERα modifications through crosstalk with signalling pathways to promote transcriptional activation and ubiquitin-mediated ERα proteolysis, with co-activators that have dual roles as ubiquitin ligases. These processes are reviewed herein. The elucidation of mechanisms whereby oestrogen drives both ERα transactivation and receptor proteolysis might have important therapeutic implications not only for breast cancer but also potentially for other hormone-regulated cancers.

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doi:10.1038/nrc3622
Corrected online
23 December 2013

Oestrogen receptor-α (ERα), which is encoded by *ESR1*, is a steroid hormone receptor superfamily member[1,2] that mediates oestrogen-stimulated proliferation in hormone-responsive cancers. ERα protein is detected in >60% of breast[3,4] and ovarian cancers[5,6] and is among the first known targets for molecular therapy in any cancer. In humans, the two receptors ERα and ERβ are encoded by different genes[7–9]. This Review focuses exclusively on ERα, and hereafter ER refers only to ERα. Oncogenic ER functions include the activation of genes that facilitate primary tumour expansion and metastasis. These include *MYC* and cyclin D1 (*CCND1*), which drive G1 cell cycle progression[10]; *BCL2* (REF. 11) and *BCLXL* (also known as *BCL2L1*)[12], which prevent apoptosis; and interleukin-8 (*IL8*) and vascular endothelial growth factor (*VEGF*)[13], which stimulate angiogenesis.

After binding to oestrogen, ER dimerizes and translocates into the nucleus, where it recruits co-activators or co-repressors, as well as chromatin-remodelling factors, to oestrogen response elements (EREs) on target gene promoters to activate or repress transcription [14]. The structural and functional domains of ER are shown in FIG. 1. ER and co-regulatory proteins must bind to DNA in a highly coordinated manner in order to overcome the physical constraints of transcribing a chromatin-encased template and to ensure that genes are turned on or off spatiotemporally in response to environmental hormone levels. Many different processes ensure that these challenges are overcome. ER co-activator[15] and co-repressor[16] availability, as well as their post-translational modifications[17], determine the selectivity and the timing of target gene expression. Many ER co-activators have enzymatic activities, including acetylation, methylation, demethylation and phosphorylation[15] (FIG. 1a). Several ER co-activators also regulate ubiquitin-dependent proteolysis (BOX 1) and modify ER. This Review discusses the relevance of these processes to ER loss in breast cancer and other hormone-regulated cancers. We discuss the mechanisms by which crosstalk between ligand-bound ER and mitogenic pathways promotes binding to dual ER co-activator ubiquitin ligases. These new mechanistic insights bring into question the traditional definition of an oestrogen-responsive tissue: high receptor levels might not be required for cellular ER action. The therapeutic implications of these findings are discussed below, as is the potential relevance of these mechanisms to other steroid hormone receptor driven cancers.

Causes of ER$^-$ status in breast and other cancers

ER is arguably the most successful molecular target in the history of cancer drug discovery. However, despite the success of anti-oestrogen therapies in breast cancer, up to one-third of breast cancers do not express detectable levels of ER protein. ER-negative (ER$^-$) breast cancers are often poorly differentiated, diagnosed at a more advanced stage and are refractory

Key points

- When oestrogen binds to the oestrogen receptor (ER), the ER dimerizes and translocates into the nucleus, where it recruits co-activators or co-repressors, as well as chromatin-remodelling factors, to oestrogen response elements (EREs) on target gene promoters in order to activate or repress transcription.
- Multiple signalling pathways downstream of receptor tyrosine kinases (such as ERBB2, epidermal growth factor receptor 1 and insulin-like growth factor 1 receptor) coordinately regulate the dynamics of ER-mediated transcriptional regulation.
- The availability of ER co-activators and ER co-repressors and their post-translational modifications determine the selectivity and timing of target gene expression. Many ER co-activators have enzymatic activities, including acetylation, methylation, demethylation and phosphorylation.
- Several ER co-activators also regulate ubiquitin-dependent proteolysis and modify ER. For example, MAPK mediates ER phosphorylation at S294 and cyclin E–cyclin-dependent kinase 2 (CDK2) phosphorylates ER at S341 to prime the interaction of ER with S-phase kinase-associated protein 2, which is the substrate-recognition subunit of the SKP1–cullin 1–F-box protein ubiquitin ligase complex. This drives target gene transcription and mediates ubiquitin-dependent ER proteolysis.
- These findings provide considerable insight into the subtleties of hormone-regulated steroid receptor stability and function that could ultimately lead to novel therapeutic strategies based on the manipulation of hormone receptor stability.

to ER-targeted therapies[18]. Other oestrogen-sensitive cancers also have variability in their levels of ER protein expression. ER protein is detected in up to 60% of ovarian cancers, but anti-oestrogen treatments have been relatively unsuccessful. Most clinical trials of ER-blocking drugs or aromatase inhibitors in ovarian cancer have included low numbers of patients, treated advanced disease and many did not assay tumour ER levels[3,6,19]. Indeed, as in breast cancer, the mechanisms that account for the ER$^-$ status of up to one-third of ovarian cancers are largely unknown, and the factors that govern ER expression in endometrial cancers remain obscure.

Several mechanisms might contribute to the ER$^-$ status in breast cancer. Deletions, rearrangements and point mutations in *ESR1* are too uncommon to account for ER$^-$ breast cancer[20,21]. MicroRNA 22 (miR-22)[22], miR-222 (REF. 23), miR-221 (REF. 23), miR-206 (REF. 24) and miR-18a[25] have been shown to target *ESR1*. Increased miR-18a levels were observed more frequently in ER$^-$ than ER positive (ER$^+$) primary breast cancers (39 negative versus 132 positive cases; $P < 0.0001$)[26], but the prevalence of these miRNAs and their relevance to ER$^-$ status in breast cancer remains poorly defined. *ESR1* promoter hypermethylation is commonly observed in ER$^-$ breast cancer cell lines[27] but has been documented in only a minority of primary ER$^-$ cancers (9 out of 39 cases assayed; 23%)[20,28]. MAPK signalling appears to decrease both *ESR1* mRNA and ER protein in a subset of breast cancers[29], and a MAPK hyperactivation gene expression signature has been described that is more common in ER$^-$ cancers[30]. Despite these findings, the mechanisms that account for ER loss in the majority of ER$^-$ breast cancers have not been fully established.

Gene expression microarray studies generally report lower *ESR1* expression in ER$^-$ breast cancers[31–33]. Interestingly, a recent analysis of triple-negative breast cancers (TNBCs; ER-, progesterone receptor (PR)- and ERBB2 (also known as HER2) negative breast cancers) showed that some clinically TNBCs have a luminal A or luminal B gene-expression phenotype and express *ESR1* mRNA[34]. Notably, more sensitive quantitative real-time PCR using fresh or directly cryopreserved breast cancers showed *ESR1* mRNA expression in most ER$^-$ breast cancers (>300 ER$^-$ cases assayed)[35–37]. *ESR1* mRNA levels were highly variable: there was a significant overlap in levels among 200 ER$^-$ and 50 ER$^+$ primary breast cancers, and in general mRNA levels were lower in ER$^-$ cancers[36]. Similar results were reported from a quantitative PCR (qPCR) analysis of >800 paraffin-embedded breast cancer sections, which showed overlapping *ESR1* mRNA values between ER$^+$ and ER$^-$ samples and a lower mean value in ER$^-$ cancers[37]. These data seem to conflict with results obtained using the 21 gene-based qPCR Oncotype DX (Genomic Health Inc.) analysis from formalin-fixed paraffin-embedded (FFPE) tissues, which showed a high concordance between ER status that was established by immunohistochemistry (IHC) and PCR-based *ESR1* mRNA quantification (*ESR1* is one gene in the Oncotype DX panel) in >20,000 reported cases[38–42]. Notably, although the reported concordance between ER IHC and *ESR1* mRNA quantification is high, up to 14% of cases that were ER$^-$ by IHC showed *ESR1* mRNA levels similar to those in ER$^+$ cancers on Oncotype DX analysis[42]. The use of different ER primers can substantially affect *ESR1* mRNA quantification by both qPCR and gene expression array[43]. Methods also differ in sensitivity. Fresh tissue gives *ESR1* mRNA values that range over seven orders of magnitude (10,000,000-fold) when using qPCR[35,36,44] but only approximately two orders of magnitude (100-fold) when using gene expression array, whereas analysis of FFPE tissue by Oncotype DX detects *ESR1* mRNA levels over only a 3,000-fold range[42]. Despite these differences, it is clear that at least a portion of ER$^-$ cancers express *ESR1* mRNA without detectable levels of protein. Because the most-sensitive methods detect overlapping *ESR1* mRNA levels in both ER$^+$ and ER$^-$ tumour types, and less-sensitive methods detect *ESR1* mRNA in up to 14% of ER$^-$ cancers (as determined by IHC)[42], ER levels in breast cancer are clearly subject to post-transcriptional and/or post-translational controls[35,36].

The following sections focus on an under-appreciated mechanism that might account for an important proportion of ER$^-$ cancers: those in which the *ESR1* gene is expressed but in which the protein cannot be detected, potentially owing to the coupling of ER target gene transcription with receptor proteolysis. ER protein levels need not be increased for its physiological roles to be manifested. ER transcriptional activity might actually be coupled to receptor turnover, and a rapid ER turnover might be required to maintain its transcriptional activity for at least a subset of target genes.

Oestrogen-ER signalling crosstalk

ER has profound effects on growth, differentiation and function in male and female reproductive systems, and it is an important regulator of bone density, brain function and cholesterol mobilization[45]. Rapid crosstalk between ER and signalling kinases occurs in breast, bone,

endometrium, brain and muscle tissues[46], as shown in FIG. 2 and summarized in TABLE 1. Kinase activation by ligand-bound ER can alter ER phosphorylation and thereby modulate receptor activation.

Ligand binding to ER rapidly activates SRC, MAPK and PI3K signalling. Oestrogen–ER binding promotes the rapid, transient interaction of ER with the signalling kinase SRC (reviewed in REFS 17,47) to activate RAS and MAPK[48–50]. Ligand-bound ER binds to the cytoplasmic portion of insulin-like growth factor 1 receptor (IGF1R), which then recruits SHC1 to activate SRC[49,51]. In some cells, oestrogen-bound ER binds to SRC and PI3K complexes, leading to AKT and MAPK activation[52]. In most breast cancers, the pathways that are activated by ligand-bound ER crosstalk are themselves subject to oncogenic activation by mutation or gene amplification of upstream receptors (including epidermal growth factor receptor (EGFR)[53], ERBB2 (REF. 54) and IGF1R[55]), RAS activation, *PIK3CA* mutation and *PTEN* deletion[56,57]. Thus, a ligand-bound ER meets a 'loaded gun' in many breast cancers, and crosstalk with oncogenic signalling is crucial for the tumour-promoting effects of oestrogen-driven gene expression in cancers.

ER phosphorylation stimulates transcriptional activity. Post-translational modifications of ER influence the stability, subcellular localization, transcriptional activity and hormone sensitivity of the ligand-activated ER-transcriptional apparatus[15,58,59]. Approximately 29 sites on the ER undergo either phosphorylation, methylation, acetylation, sumoylation, palmitoylation or ubiquitylation[17]. Ligand-bound ER activates mitogenic signalling kinase cascades to drive rapid non-genomic mitogenic effects that include the phosphorylation of ER and its co-activators. These feedforward loops augment ER transcriptional activity[60–63].

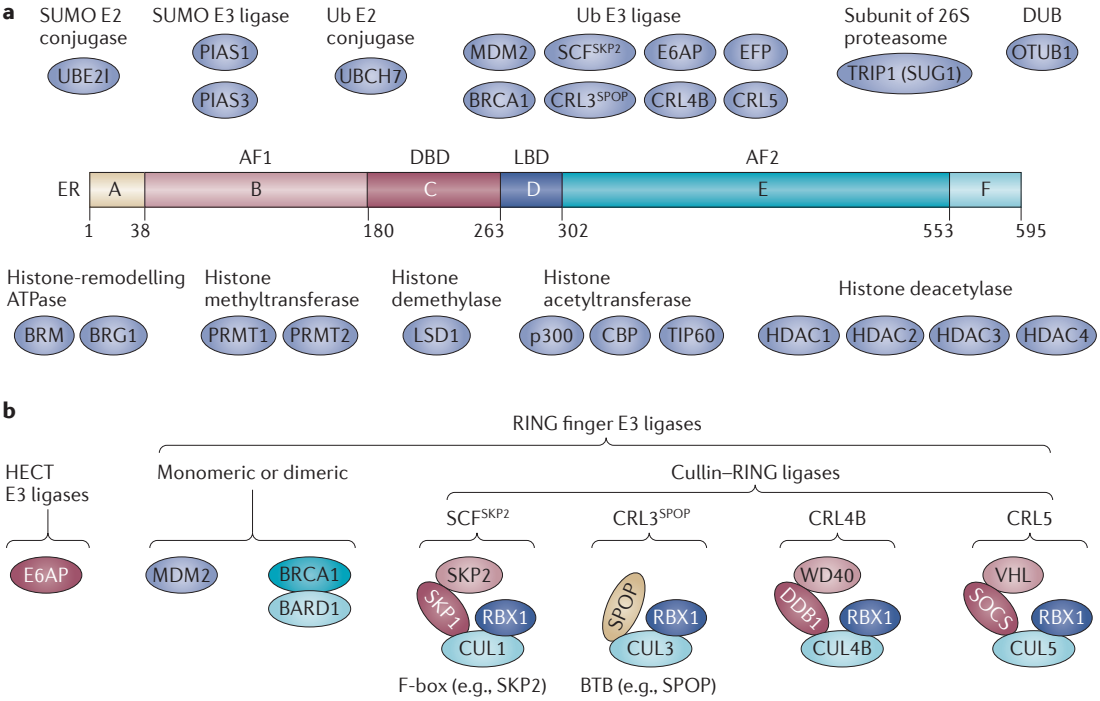


Figure 1 | **ER co-activators and ER E3 ligases. a** | The figure represents the structure of the oestrogen receptor (ER) and ER co-activators. The 595-amino-acid receptor has six nuclear receptor structural domains (A–F) that include activation function 1 (AF1) and AF2 domains, a conserved DNA-binding domain (DBD) and a ligand-binding domain (LBD). Major enzymatic ER co-activators and co-repressors are also shown. Ubiquitin (Ub)-conjugating enzymes (E2) and ubiquitin ligases (E3), histone methyltransferases and acetyltransferases have co-activator roles during ER-mediated transcription, whereas enzymes such as deubiquitylases (DUBs), small ubiquitin-like modifier E2 (SUMO E2) and SUMO E3 proteins, as well as histone demethylases and histone deacetylates (HDACs) are generally co-repressors. **b** | E3 ubiquitin ligases that co-activate ER are summarized and organized by subfamilies. Many of these proteins regulate ER stability, but their transactivation effects and priming phosphorylation events are unknown. BARD1, BRCA1-associated RING domain 1; BRM, brahma; BRG1, brahma-related gene 1; CBP, CREB-binding protein; CRL3, cullin–RING ligase 3; CUL 1, cullin 1; DDB1, DNA damage-binding protein 1; E6AP, E6-associated protein; EFP, oestrogen-inducible RING finger protein; HECT, homologous to E6AP C terminus; LSD1, lysine-specific demethylase 1; OTUB1, OTU domain-containing ubiquitin aldehyde-binding protein 1; PIAS1, protein inhibitor of activated STAT protein 1; PRMT1, protein arginine methyltransferase 1; RBX1, RING-box protein 1; SCF, SKP1–CUL1–F-box protein complex; SKP2, S-phase kinase-associated protein 2; SOCS, suppressor of cytokine signalling; SPOP, speckle-type POZ protein; TIP60, 60 kDa TAT-interactive protein; TRIP1, thyroid hormone receptor-interacting protein 1; UBE2I, ubiquitin-conjugating enzyme E2 I; VHL, von Hippel–Lindau.

These phosphorylation events modulate ER function by altering its binding to ligand, to target gene promoters, or to ER co-activators[17]. Important ER phosphorylation sites are summarized in TABLE 2.

Ligand-activated ER proteolysis

The role of the ubiquitin-proteasome in transcriptional regulation has recently gained prominence. For many transcription factors, including nuclear factor-κB (NF-κB), JUN, MYC, general control protein GCN4 and E2F1, phosphorylation events and protein–protein interactions that stimulate transcriptional activation also trigger proteolysis of the transcription factor[64,65]. Several laboratories, including our own, have extended these observations to the hormone receptor field, including PR[66], the thyroid receptor[67], androgen receptor (AR)[68] and ER[69–71]. Of note, PR sumoylation can compete with PR ubiquitylation to represses PR transcriptional activity and retard ligand-induced PR proteolysis[72,73]. Similar mechanisms might exist for other steroid receptors.

Ligand binding activates rapid proteasomal ER degradation. ER is rapidly ubiquitylated and degraded after oestrogen binding[74–76]. Different ligands stimulate ER proteolysis by different degrees[77]. A link between the ubiquitylation of ER and its transcriptional activity was suggested by the observation that proteasome inhibitors increase ER levels but impair ER-driven transcription at certain promoters[78,79]. Ligand binding rapidly signals ER ubiquitylation, and ubiquitylated ER cycles on and off ERE promoter sites to activate target gene transcription[78,80].

Many ubiquitin proteasomal pathway components are ER co-activators[81] (FIG. 1). Ubiquitin ligases comprise two major families: homologous to E6AP C terminus (HECT)-family ligases and RING finger ligases[81]. There are 61 HECT-family ligases and >1,000 RING finger ligases in mammals. Approximately 50% of RING finger E3 ligases are multidomain proteins that ubiquitylate substrate on their own or with the help of a single partner, such as MDM2 or BRCA1–BARD1 (BRCA1-associated RING domain 1). The remaining RING finger E3 ligases are multiprotein complexes and include the cullin (CUL)–RING ligases. Ubiquitin ligases BRCA1 (REFS 82–84), MDM2 (REFS 85,86), SKP1–CUL1–F-box S-phase kinase-associated protein 2 (SCF^{SKP2})[70] and E6-associated protein (E6AP)[87] promote oestrogen-induced transcriptional activity. A number of other CUL–RING ligases have also been shown to govern ER stability. These include the CUL4B aryl hydrocarbon receptor (AHR)[88], CUL5 (REF. 89) and CUL7 (REF. 71) (FIG. 1b). In addition to the specific ubiquitin ligases that are reviewed in detail below, ubiquitin-conjugating enzyme UBCH7 (also known as UBE2L3) and the proteasome subunit, thyroid hormone receptor-interacting protein 1 (TRIP1; also known as SUG1), also function as steroid hormone receptor co-activators (reviewed in REFS 90,91). Although these different ubiquitin E3 ligases share redundant functions in terms of ER stability, data increasingly indicate that they modulate stimulus- and cell type-specific ER functions to influence the range of target genes that are activated or repressed after ligand binding.

Many ubiquitin ligases only bind to appropriately phosphorylated substrates[92]. Substrate phosphorylation is tightly regulated to ensure the proper timing and extent of ubiquitylation. Data increasingly indicate that different ER phosphorylation events, which are mediated by ligand-bound ER crosstalk with signalling kinases, might couple the receptor's transcriptional activation to its proteolytic demise. The site-specific phosphorylations of ER that promote its binding to different ER co-activator–E3 ligases are reviewed below[69–71] and summarized in FIG. 2. How ER phosphorylation events specify differences in co-activator binding to modulate broad patterns of target gene expression remain to be defined.

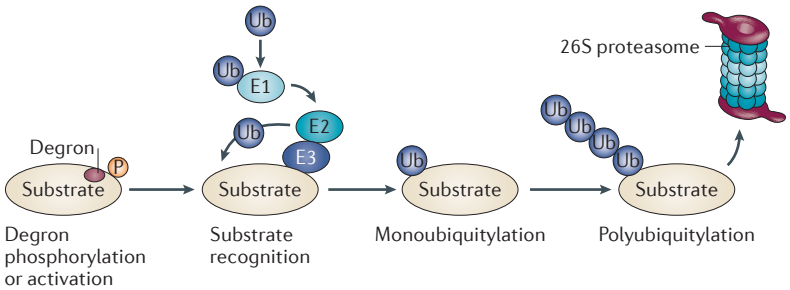
Ubiquitin ligases that co-activate ER

E6AP, MDM2, BRCA1 and SCF^{SKP2} are E3 ubiquitin ligases that have dual roles as ER co-activators (FIG. 1; FIG. 2; TABLE 3) and link ER to major pathways that govern oncogenic signalling, genomic stability and cell cycle regulation.

E6AP. E6AP, of the HECT domain E3 ligase family, mediates ubiquitin-dependent degradation of p53 in cells that are infected with the human papilloma virus[93,94]. E6AP also functions as a ligand-activated co-activator for the steroid hormone receptors ER, AR, PR and growth hormone receptor (GHR)[87,95,96], and it is co-recruited with ER to promoters that contain an ERE[69,78]. A link between E6AP and ER levels and/or activity has been genetically established: *E6ap*-null animals show increased ER protein levels in mammary tissue but defective oestrogen action, with aberrant ovulation and reduced uterine growth, compared with wild-type littermates[97]. By contrast, transgenic E6AP expression reduces ER levels in mouse mammary tissue[95].

Box 1 | **Ubiquitin–proteasome system**

For a substrate protein to be recognized by an ubiquitin (Ub) ligase, it must often be properly phosphorylated or methylated at a target sequence, which is often known as a degron. Ubiquitin is covalently attached to a substrate lysine residue by the sequential action of an ubiquitin activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) to build a polyubiquitin chain on the substrate that signals substrate targeting by the 26S proteasome for degradation (see the figure). P, phosphate.



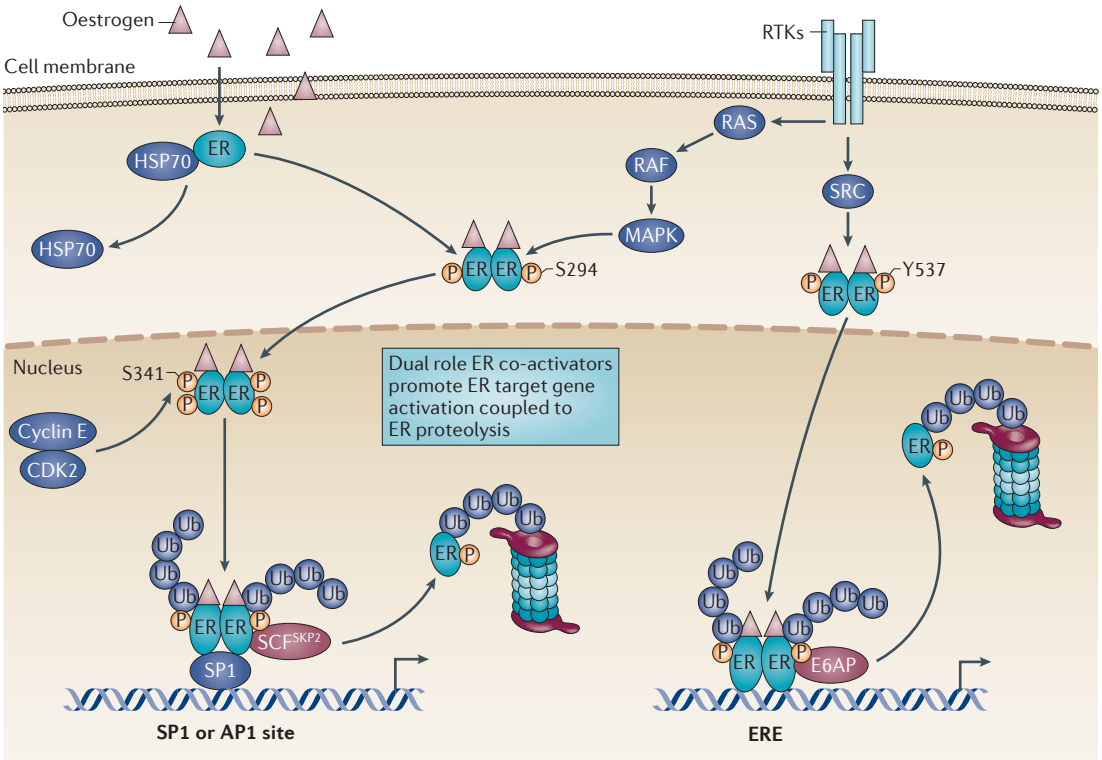


Figure 2 | **ER cytoplasmic signalling cascades and nuclear target gene expression.** Emerging evidence of the interaction between hormonal and growth factor signalling pathways is shown. These multiple signalling pathways downstream of receptor tyrosine kinases (RTKs; such as ERBB2, epidermal growth factor receptor and insulin-like growth factor 1 receptor) coordinately regulate the dynamics of oestrogen receptor (ER)-mediated transcriptional regulation. Several regulatory molecules and their interacting proteins are shown. MAPK mediates ER phosphorylation at S294, and cyclin E–cyclin dependent kinase 2 (CDK2) phosphorylates ER at S341 to prime the ER–S-phase kinase-associated protein 2 (SKP2) interaction (shown on the left side of the figure). SRC-mediated ER phosphorylation at Y537 primes the ER–E6-associated protein (E6AP) interaction to drive target gene transcription (shown on the right side of the figure). Both E6AP and SKP1–CUL1–F-box S-phase kinase-associated protein 2 (SCF^{SKP2}) also mediate ubiquitin-dependent ER proteolysis. ERE, oestrogen response element; HSP70, heat shock protein 70; P, phosphate; Ub, ubiquitin.

Recent work showed that SRC accelerates oestrogen-dependent ER proteolysis[36]. Oestrogen stimulates rapid SRC activation, and SRC phosphorylates ER at Y537 to facilitate its binding to E6AP. This complex is then recruited to a subset of ER target gene promoters, leading to their transcriptional activation[69]. The interaction of ER with E6AP also catalyses rapid ER ubiquitylation in biochemical assays and in cells. Furthermore, the expression of a mutant (Y537F) ER results in increased ER stability but reduced binding to E6AP and reduced target gene activation. This study was the first to indicate that ER crosstalk with a specific kinase (SRC in this case) could mediate ER phosphorylation to promote the recruitment of a dual-role co-activator that also drove ER degradation (FIG. 2). Although other studies have reported that ER Y537F is functional in ER luciferase assays, such studies did not take into account the increased steady-state levels of ER Y537F when considering its transcriptional efficiency[98,99]. These data support a model in which ER transcriptional activation can be coupled to receptor degradation as a mechanism to fine-tune ER action. The possibility that Y537 phosphorylation could also modulate the interaction of ER with other ubiquitin ligase ER co-activators, global ER target gene promoter selection and transcriptional activation or repression remains to be explored. This work suggests that receptor action and receptor levels are not synonymous. After ligand binding, ER transcriptional activity is maintained despite ongoing proteolysis and decreasing ER levels, introducing the possibility that hormonally sensitive tissues may not always have readily detectable levels of ER protein.

MDM2. MDM2 is a single-subunit RING finger E3 protein that has a key role in oncogenesis because it contributes to p53 proteolysis[100]. This multifunctional protein also promotes ER-mediated transcription and receptor proteolysis. Overexpression of MDM2 often occurs in breast cancer tissue and cell lines, but has not been shown to inversely correlate with ER levels. MDM2 functions as an ER co-activator[86] and can directly interact with ER in a ternary complex with p53 to regulate ER turnover[85]. Oestrogen activates the cyclic co-recruitment of MDM2 and ER to the ERE motif of the trefoil factor 1 (*TFF1*; also known as *PS2*) promoter[78]. MDM2 was recently shown to bind to ER and increase ER–SP1-mediated transcriptional activation in MCF-7 and ZR-75 breast cancer cells[101]. To date, the spectrum

Table 1 | **Rapid crosstalk of ER with MAPK, PI3K or SRC in a tissue-specific manner**

Tissue	Pathways	Physiological function	Refs
Breast	MAPK	Cell proliferation	51,151
	IGF1R–PI3K–AKT	Mitogenesis	52,152
Bone	SRC–SHC–ERK	Anti-apoptosis	153
	PKCα and SRC	Osteoblast differentiation	154
Endometrium	MAPK	Pro-inflammatory cytokine expression in lumen formation and maintenance	155
	PI3K	Anti-apoptosis	156
Pituitary	Calcium flux	Release of prolactin	157
Neurons	MAPK	Protection from reactive oxygen species	158,159
Muscle	MAPK	Anti-apoptosis	160
	PI3K	Expression of calcium channel gene	161
Vascular endothelium	PI3K–AKT and MAPK	Induction of endothelial nitric oxide synthase activation	162

ER, oestrogen receptor; IGF1R, insulin-like growth factor 1 receptor; PKCα, protein kinase Cα; SHC1, (SRC homology 2 domain containing) transforming protein 1.

of ER target genes that are governed by the MDM2–ER interaction remains unknown. Furthermore, the relevance of this interaction to hormone-regulated cancers and its potential as a target for therapeutic intervention has not been explored.

BRCA1. Germline mutations in *BRCA1* predispose individuals to familial breast and ovarian cancers[102], and BRCA1 is involved in DNA repair[103]. BRCA1 binds to ER, and this complex has been postulated to have a role in DNA damage repair[104,105]. BRCA1 can function as a transcriptional regulator[106], but it also binds to BARD1 to form a dimeric RING finger E3 ubiquitin ligase. Several lines of evidence suggest that BRCA1 functions as an E3 ligase for ER[82,83]. ER is an *in vitro* substrate for the BRCA1–BARD1 ubiquitin ligase, and cancer-predisposing *BRCA1* mutations that affect the RING motif abrogate its *in vitro* E3 ligase function towards ER[83,107].

Although BRCA1–BARD1 can function as an E3 ligase *in vitro*, the effects of BRCA1 on ER transcriptional activity are controversial. It can both co-repress[84] and co-activate ER-mediated transcription in different cellular contexts[108]. BRCA1 can function as a co-repressor of ER-mediated transcription, but the ectopic overexpression of either p300 or CREB-binding protein (CBP) reverses the inhibition of ER activity by BRCA1 (REF. 108). Additional research has shown that oestrogen-bound ER recruits BRCA1 into a transcriptional activation complex that contains the co-activator CBP[109], but the subset of ER target genes that are co-regulated by BRCA1 has not been fully defined. BRCA1 appears to function as either a co-activator or a co-repressor of other steroid receptors in different cellular contexts[110]. Indeed, BRCA1 is a well-defined AR co-activator[111,112].

Most *BRCA1*-mutant breast cancers are ER^- (REF. 113). This has been postulated to result from transcriptional repression of *ESR1* by mutant BRCA1 (REF. 114), whereas wild-type BRCA1 transactivates *ESR1*. Interestingly, oestrogen action appears to contribute to breast cancer development in *BRCA1*-mutant carriers, since the risk of BRCA1-mutant breast cancer is decreased by prophylactic oophorectomy and by tamoxifen[113,115,116]. BRCA1 may serve a dual role as a co-activator and E3 ligase for ER to mediate constitutive oestrogenic action, coupled to ER loss. This warrants further investigation since it would have substantial therapeutic implications.

SKP2. SCF complexes comprise a large E3 ligase family that has a prominent role in cell cycle regulatory protein degradation[117]. In humans, 70 different F-box proteins govern SCF E3 substrate specificity[118]. Among them, SKP2 is oncogenic and overexpressed in many human cancers[71,119–123], and it degrades the cell cycle inhibitor p27 (also known as CDKN1B)[124].

Two groups recently reported a dual role for SCF^{SKP2} as an ER co-activator–E3 ligase[70,71]. Both groups showed that SKP2 overexpression or knockdown modulates ligand-activated ER degradation in cells. The F-box protein SKP2 was shown to bind to ER through a signature LXXLL co-activator motif at amino acids 248–252 (REF. 70). Although an initial report proposed that ER phosphorylation at S294 by a MAPK-dependent pathway primes its ubiquitylation by SCF^{SKP2} (REF. 71), further evidence indicates that priming phosphorylation of ER at S341 by cyclin E–cyclin-dependent kinase 2 (CDK2) increases SKP2-mediated ER ubiquitylation and proteolysis *in vitro*[70]. Loss of the potential to phosphorylate ER at an SKP2-binding ExS motif that surrounds ER S341 impaired both ER–SKP2 binding and cyclin E–CDK2-mediated priming of SKP2-driven ER proteolysis *in vitro*. ER–SKP2 complexes were absent in oestrogen-deprived quiescent cells, and their levels increased to maximum in late G1 phase or early S phase, which was coincident with the increase in SKP2 levels[70,71]. Notably, ER–SKP2 binding is biphasic: oestrogen stimulates early rapid MAPK activation and

low-level ER–SKP2 binding, which is followed by a larger increase in ER–SKP2 binding after cyclin E–CDK2 activation in late G1 phase[70]. These data permit a model in which ER phosphorylation by MAPK at S294 primes its subsequent phosphorylation by cyclin E–CDK2 at S341, which would promote further SKP2 recruitment to potentiate the activation of ER target genes in late G1 and S phases (FIG. 2).

ER–SKP2 binding in late G1 phase is coupled to a novel mechanism of late activation of ER target genes, in which gene induction occurs several hours after ligand stimulation. Expression profiling identified oestrogen-induced genes that are activated at the G1-to-S phase transition. Oestrogen also stimulated ER, SKP2, SRC3 (also known as AIB1) and RNA polymerase II (Pol II) occupancy at *E2F1* and *BLM* promoters and dramatically induced these genes in the late G1 phase, with activation peaking 12–18 hours after ligand addition[70].

SKP2-dependent ER transactivation of *E2F1* provides a feedforward mechanism to drive S phase entry. E2F1 is a known transactivator of *CCNE1* (which encodes cyclin E1) and *CCNA1* (which encodes cyclin A1). The oestrogen-activated increase in E2F1 expression, together with cyclin D–CDK-mediated and cyclin E–CDK-mediated inactivation of the E2F1 repressor retinoblastoma protein (RB), would feed forward to further induce the transcription of *SKP2*, *CCNE1*, *CCNA1* and other E2F1 target genes that drive S phase and G2/M phase progression[125]. Thus, SCF^{SKP2} serves as a dual E3 ligase and ER co-activator to drive late induction of ER target genes, several hours after ligand stimulation to mediate late cell cycle events.

Other E3 ligases and deubiquitylation enzymes that modulate ER function. Recent studies have also shown that at least three other E3 ligases and deubiquitylases (DUBs) have important roles in fine-tuning ER activity.

The complex CUL3–speckle-type POZ protein (SPOP) is a CUL3–RING finger ligase (CRL3) family member[126] that mediates ER ubiquitylation in cells[127] and can also ubiquitylate and degrade an important ER co-activator, SRC3 (REF. 128). Thus, the CUL3–SPOP complex controls the levels of both ER and the ER co-activator SRC3, and hence ER-mediated transcription.

CUL4B binds to DNA damage-binding protein 1 (DDB1) as well as to DDB1- and CUL4-associated factor (DCAF) to form a CUL4B–RING ubiquitin ligase (CRL4B).

Table 2 | **Breast cancer-relevant ER phosphorylation regulates ER stability and/or activity**

Phosphorylation site	Kinase	Function	Refs
S46 and S47	PKC	Increases transcriptional activation	163
Y52	ABL	Increases transcriptional activation and invasion	164
S104, S106 and S118 (REFS 62,149,165)	CDK2	Increases transcriptional activation	166
	CDK7	Increases transcriptional activation	167
	MAPK	Tamoxifen resistance, transcriptional activation	168,169
	IKKα	DNA binding and co-activator recruitment	170,171
S167	$p90^{RSK}$	Anti-apoptosis; increases DNA binding and transcriptional activation; increases SRC3 recruitment	168,172
	AKT	Tamoxifen resistance; increases transcriptional activation	60
	IKKε	Tamoxifen resistance	173
S212	Not determined	Increases transcriptional activation	174
Y219	ABL	Increases transcriptional activation and invasion	164
S236	PKA	Decreases DNA binding and transcriptional activation	175,176
S282	CK2	Decreases transcriptional activation	163,177
S294	MAPK	Promotes SKP2 binding and ER proteolysis, possibly by priming ER phosphorylation by CDK2 at S341; increases transrepression of *CDKN1A*	70,71
	CDK2	Primes ER phosphorylation at S118 and S167	178
S305	PAK1	Tamoxifen resistance; increases transcriptional activation	179,180
	PKA	Prevents K303 acetylation	181
		Tamoxifen resistance; increases SRC1 recruitment	182
T311	MAPK	Increases nuclear import and increases transcriptional activation	177,183
S341	CDK2	Increases SKP2 recruitment, ER proteolysis and transcriptional activation	70
Y537	SRC	Increases E6AP recruitment, ER proteolysis and transcriptional activation	69,184
S559	CK2	Decreases transcriptional activation	163

CK2, casein kinase 2; CDK, cyclin-dependent kinase; *CDKN1A*, CDK inhibitor 1A; E6AP, E6-associated protein; ER, oestrogen receptor; IKK, inhibitor of NK-κB kinase; $p90^{RSK}$, 90 kDa ribosomal protein kinase; PAK1, p21-activated kinase 1; PKA, protein kinase A; SKP2, S-phase kinase-associated protein 2.

This complex can bind to AHR[129]. Dioxins, which bind to AHR, were shown to modulate oestrogen signalling, in part by stimulating the direct association of AHR with ER[88]. Agonist-bound AHR and ER work together to regulate target gene expression. In this complex mechanism, the AHR co-regulatory complex CUL4B–AHR also promotes ER ubiquitylation and degradation. Whether the ensuing ubiquitylation of the ER has a conformational role in the transcriptional activation of ER has not been established.

CUL5 (also known as VACM1) belongs to the CRL5 subclass of CUL–RING ubiquitin E3 ligases. CUL5 was shown to mediate proteasomal degradation of ER in the T47D breast cancer cell line[130]. In each of the cases discussed above, ER proteolysis limited the transcriptional function of the receptor through its degradation. None of these interactions (with CRL3, CRL4B or CRL5) has been shown to function directly in ER co-activation.

Paradoxically, a deubiquitylating enzyme that opposes E3 action by removing polyubiquitin from the ER was also found to regulate ER stability and activity. The OTU domain-containing ubiquitin aldehyde-binding protein 1 (OTUB1) belongs to the ovarian tumour cysteine protease (OTU) DUB subfamily[131]. OTUB1 was recently reported to regulate the availability and functional activity of ER in Ishikawa cells and to decrease ER transcriptional activity by stabilizing chromatin-bound ER protein in an inactive state[132].

Implications for target gene regulation

The relationships between the control of transcription and transcription factor degradation are intriguing. The above examples reveal the importance of ER ubiquitin ligase co-activators to the dynamics of ER transcriptional activity and receptor stability. The intimate coupling between the activation of transcription by ER and ER proteolysis is counterintuitive because the very processes that mediate receptor activation, in some cases also limit its extent. Data presented in this Review support a model in which ligand-activated, properly phosphorylated ER would bind to and recruit an E3 ligase co-activator together with other cofactors and Pol II at certain promoters to form a transcriptional initiation complex (FIG. 3). Ubiquitylation (monoubiquitylation or polyubiquitylation) would induce conformational changes that are necessary for the recruitment of other co-activators or chromatin modifiers and for transcriptional competence. Proteasome-mediated receptor degradation also appears to regulate co-activator and co-repressor exchange[133], to initiate complex disassembly and to facilitate the transition to a productive elongation complex and transcriptional elongation. Paradoxically, ER–E3 cofactor binding rapidly converts activated ER to an inactive state by recruiting the proteasome to clear the expended ER–E3 cofactor complex. The clearance of ER from the promoter paves the way for another round of ER–E3 cofactor binding, thus permitting effective and continuous cycles of promoter firing[134] (FIG. 3). In addition, ER proteolysis might provide an efficient regulatory checkpoint that is linked to environmental hormonal signals[135]. Cyclic binding and degradation would ensure that subsequent transcription cycles only continue in the presence of an adequate level of hormone. The ER would be removed from a promoter when the hormone signal is lost. The spatiotemporal interaction of ER with ER ubiquitin ligase-containing co-activators has not been fully elucidated. Further work in this area is needed to resolve these fundamental issues in receptor biology and transcriptional regulation.

Anticancer therapy with ER and kinase inhibitors

Implications for ER⁺ cancers. The identification of E3 ligase ER co-activators has implications for ER⁺ breast cancers and might prove to be relevant to ovarian cancer and other cancers in which ER is expressed at variable levels. A better understanding of the signalling mechanisms that drive ER action will permit further development of therapies that target the ER co-activator interaction. In ER⁺ cancers, SKP2 might substantially contribute to mitogenic ER function by transactivating genes that promote G1-to-S phase progression.

Combinations of anti-oestrogens and signalling kinase inhibitors have been attractive for clinical development because of the independent mitogenic effects

Table 3 | **ER E3 ligase as an ER transcription cofactor**

E3 ligase family	Substrate-recognition protein	ER co-regulator function	Refs
HECT			
Monomeric	E6AP	Co-activator	69
Monomeric	CHIP	Not known	185
RING			
Monomeric	MDM2	Co-activator	86
Dimeric	BRCA1–BARD1	Co-repressor	83,84
Multimeric SCF	SKP2	Co-activator	70
Multimeric CRL3	SPOP	Not known	127
Multimeric CRL4B	AHR	Not known	88
Multimeric CRL5	Not determined	Not known	130

AHR, aryl hydrocarbon receptor; BARD1, BRCA1-associated RING domain 1; CHIP, carboxyl terminus of HSP70-interacting protein; CRL, cullin–RING finger ligase; E6AP, E6-associated protein; HECT, homologous to E6AP C terminus; ER, oestrogen receptor; SCF, SKP1–CUL1–F-box protein complex; SKP2, S-phase kinase-associated protein 2; SPOP, speckle-type POZ protein.

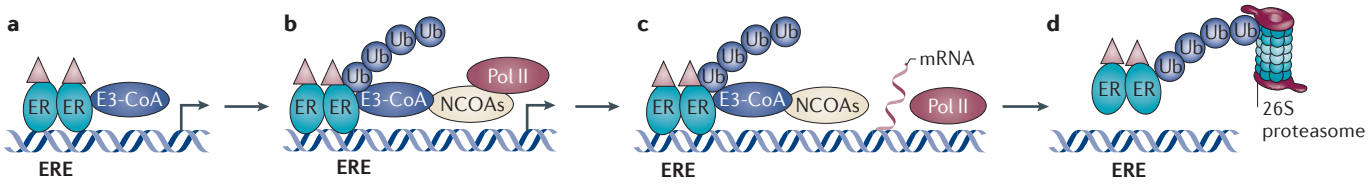


Figure 3 | **A model for ER proteolysis-coupled transcriptional activations of target genes.** Increasing data support a model in which oestrogen-bound oestrogen receptor (ER) is recruited to a subset of target gene promoters, along with dual-role E3 ligase and ER co-activators that promote ER-driven gene expression through a mechanism that is coupled to receptor proteolysis. **a** | Target gene activation starts with oestrogen-bound ER undergoing a phosphorylation event that promotes E3 ligase binding and facilitates binding of the complex to oestrogen response element (ERE) or to AP1 or SP1 sites at a target gene promoter. **b** | ER ubiquitylation might alter the receptor conformation to facilitate pre-initiation complex assembly. **c** | When transcription initiation is complete, ubiquitylation marks ER for degradation and might help to disassemble the transcription initiation complex, which facilitates the transition to a productive elongation complex and elongation of transcription. **d** | ER clearance by proteolysis would permit promoter recharging and thereby allow the next round of promoter firing. The process shown in part **c** would facilitate continuous and sensitive ER responses to cellular oestrogen concentrations and co-activator availability. E3 ubiquitin ligase inhibition or proteasome inhibitor drugs would prevent receptor clearance from the ERE and thus block the next round of promoter firing. E3-CoA, ubiquitin ligase-ER co-activator; NCOA, nuclear receptor co-activator; P, phosphate; Pol II, RNA polymerase II; Ub, ubiquitin.

of both oestrogens and receptor tyrosine kinases[136]. The role of signalling kinases as activators of steroid hormone receptor-mediated transcription provides an additional rationale for these drug combinations. In preclinical ER^+ breast cancer and ovarian cancer models, dual targeting with SRC and ER blocking drugs showed synergistic anticancer efficacy in cells and *in vivo*[19,137–139]. These results have encouraged the further pre-clinical and clinical development of therapies that combine SRC, MEK or CDK2 inhibitors with anti-oestrogens to abrogate the effects of ER co-activator E3 ligases on ER. Targeting ER co-activators (such as SCF^{SKP2} (REFS 140,141)) or the priming kinases such as SRC, might add to the therapeutic effects of selective oestrogen receptor modulators (SERMs) and aromatase inhibitors. The identification of ER target gene expression signatures that are associated with specific ER co-activator recruitment might ultimately guide the clinical application of co-activator inhibitors in anticancer therapy.

Implications for ER⁻ cancers. The implication of ER regulation by dual-role co-activator E3 ligases is that certain tissues might be hormone sensitive and have constitutive receptor activity in the context of relatively low or potentially undetectable ER protein levels. In certain tumour tissues, ER protein levels may be low, but the receptor itself may be disproportionally activated as a result of transcription-coupled degradation. SRC, MAPK and cyclin E–CDK2 can all regulate liganded ER activity in a manner that does not solely depend on steady-state ER levels. ER activation-coupled proteolysis may occur in several hormonally regulated cancers, including ER^- breast, ovarian and endometrial cancers, certain forms of colon cancer and malignancies of the bone and the brain. Ligand-bound ER crosstalk with signalling kinases, including MEK, MAPK, CDK2 and SRC, might predicate different promoter selection in different tissue contexts. Crosstalk with different oncogenically activated signalling kinases could phosphorylate hormone receptors at different sites, including ER S294, S341 and Y537, to alter co-activator or co-repressor binding, modify chromatin conformation and drive different patterns of target gene expression[69–71]. A subset of ER^- breast cancers and even normal tissues might prove to be regulated by oestrogen: these would express *ESR1* mRNA but have low ER protein levels owing to accelerated ER proteolysis. These concepts provide a new way of viewing hormone-sensitive physiology in tissues with low or undetectable hormone receptor levels.

The corollary of this is that the efficacy of anti-oestrogen therapy might not be solely dependent on ER protein levels. Although abundant epidemiological evidence shows that ER^- breast cancers do not respond to tamoxifen or aromatase inhibitors[3,4,142], the possibility that sensitivity to anti-oestrogens might be enhanced or restored by targeting the ER degradation process warrants further study. SCF^{SKP2} might drive ER loss in a subset of ER^- cancers. Levels of activated SRC and SKP2 are inversely correlated with ER protein levels in human breast cancers, and overexpression of these proteins is associated with poor prognosis[36,71,123]. Bhatt *et al.*[71] confirmed the inverse relationship between ER and SKP2 in human breast cancers and showed that downregulation of SPK2 in ER^- breast cancer cell lines increased ER levels and restored responsiveness to anti-oestrogen therapy. A link between MAPK activation and ER loss has been shown and, in certain ER^- cell lines and *ex vivo* tumour cultures, MAPK inhibition was shown to restore ER levels and sensitivity to anti-oestrogens[29,71,143,144]. Taken together, these data suggest that constitutive ER proteolysis could drive ER loss in certain ER^- cancers. In these, proteasome inhibitors or drug-mediated inhibition of kinases that prime ER for degradation might have the potential to restore both ER levels and responsiveness to ER blockade or aromatase inhibitors[29,71].

Implications for prostate cancer. The concept that hormone receptor transcriptional activation might be coupled to its proteolysis could also have implications for prostate cancer. It was previously thought that AR was

stabilized by ligand binding and that the proteasome only served only to reduce AR levels and function[145]. This concept was challenged by a report indicating that the proteasome is required for AR transcriptional activity[146] and that the ubiquitin ligase MDM2 co-activates AR at the prostate-specific antigen (*PSA*; also known as *KLK3*) promoter and promotes AR ubiquitylation and degradation[68]. Moreover, another AR co-activator, SIAH2, has also been shown to function as an AR ubiquitin E3 ligase[147]. SIAH2 and MDM2 might have distinct roles that govern both AR turnover and the activation of a subset of AR target genes[147]. During prostate cancer progression, a decrease in AR levels might not always indicate androgen independence, but in some cases reflect an increase in sensitivity to androgen that is due to AR activation-coupled proteolysis. The possibility that targeting the proteasome might also impair AR-dependent oncogenic transcriptional activity could yield new strategies for therapeutic intervention in this disease.

Conclusions

For many transcription factors, activation is linked to proteolytic degradation[65]. Recent advances have identified mechanisms that link the transcriptional activity of ER with its proteolysis. ER activation in human cancers is promoted by crosstalk between ER and oncogenically activated kinases, including receptor tyrosine kinases, PI3K, SRC and MAPK[69,148,149]. SRC and MAPK can also activate ER turnover in breast cancer models[29,36,69,150]. Y537 phosphorylation of ER by SRC recruits E6AP as an ER ubiquitin ligase[69], whereas serine phosphorylations of ER by MAPK or CDK2 recruit another ubiquitin ligase, SCF^{SKP2} (REFS 70,71). The distinct ER target gene expression profiles that are predicated by different E3 ligase–ER co-activators remain to be defined. Importantly, the study of ER co-activator–E3 ligases in human cancer cell lines and primary tumours has informed our understanding of steroid hormone receptors in general. The concept that at certain promoters, receptor activation is coupled to receptor proteolysis might prove relevant to all steroid hormone receptors.

In addition to providing new insight into the subtleties of hormone-regulated steroid receptor stability and function, studies of 'activation-coupled proteolysis' could lead to novel therapeutic strategies that modulate hormone receptor stability. These findings introduce the possibility that cancer growth could be controlled by targeting specific ubiquitin E3 ligases or using proteasome inhibitors, such as bortezomib, to restore AR and ER levels in selected prostate, breast and ovarian cancers, either alone or combined with SRC or MEK inhibitors. Elucidation of hormone receptor activation-coupled proteolysis might open new avenues for molecular-targeted therapy in hormone-regulated cancers.

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Acknowledgements
This work was supported by US National Institutes of Health grant R01CA123415 (J.M.S.) and by US Department of Defense pre-doctoral grant W81XWH-11-1-0097 (W.Z.). The authors thank M. Lippman, D. El-Ashry, Z. Nawaz, C. Perou and laboratory members for helpful discussions. The authors apologize for omissions in citations and coverage: strict space and citation limits preclude the inclusion of many important past and recent works.

Competing interests statement
The authors declare no competing interests.

Appendix 3 Book Chapter 2011

Cross Talk Between ERα and Src Signaling and Its Relevance to ER Status and Hormone Responsiveness

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Abstract While two thirds of breast cancers are ER positive and a majority of these are responsive to endocrine therapies, up to one third of newly diagnosed breast cancers lack detectable ER protein. ER negative breast cancers are thought to be resistance to endocrine therapy. Here we review several potential mechanisms underlying the ER negative status of these breast cancers. The role of cross-talk between ER and Src-activated signal transduction as a mediator of both ER proteolysis and ER transactivation is discussed. Src kinase is often activated in breast cancer. Liganded ER rapidly and transiently activates Src which phosphorylates ER. For a subset of ER-responsive promoters, ER phosphorylation by Src leads to enhanced ER binding to the promoter, increased interactions with E3 ubiquitin ligases, and rapid ER degradation, in a process in which ER activation is coupled to its degradation. Thus, the function of ER may not be solely dependent

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G. Castoria and A. Migliaccio (eds.), *Advances in Rapid Sex-Steroid Action*,
DOI: 10.1007/978-1-4614-1764-4_4,

on the steady state levels of ER protein. A subset of ER negative breast cancers that have ER mRNA but lack detectable ER protein levels may ultimately prove to be responsive to estrogen. These observations may have broader implications for estrogen driven gene expression. Cells of estrogen responsive tissues (ovary, bone, brain and intestine) could have low ER protein levels, but retain responses to estrogen through estrogen driven ER proteolysis-coupled transcriptional activity.

Keywords Estrogen receptor · Src kinase · Breast cancer · Signal transduction · Ubiquitin · Proteolysis

Abbreviations

EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
Her 2	Human epidermal growth factor receptor 2
IGF	Insulin growth factor
IGF-IR	IGF-I receptor
MAPK	Mitogen activated protein kinase
MEK	Mitogen-activated protein (MAP) kinase kinase
PI3K	Phosphatidylinositol 3-kinase
Ras	Rat sarcoma

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1 Estrogen Receptor in Breast Cancer

Estrogen regulates proliferation of many cell types expressing its cognate receptors and is a risk factor for breast cancer development. Estrogen exerts its biological functions through binding to its intracellular receptors, the estrogen receptors, ERα and ERβ, which are members of the nuclear hormone receptor superfamily [1]. The two different types of ER identified in humans, ERα and ERβ are encoded by different genes [2–4]. ERα is expressed in the epithelium of the breast, endometrium, ovary, bone and brain in the adult human [2]. ERβ is widely expressed throughout the body. ERα co-exists with ERβ in the mammary epithelium, uterus, adipose tissue, skeletal muscle, liver, pancreas and the central nervous system. ERβ is also expressed in ERα-negative tissues including the prostatic and pulmonary epithelium [5]. While ERβ is expressed in some breast cancers, the prognostic implications of this have not been fully defined [6]. The vast majority of studies of ER in breast cancer pertain to ERα [7]. Since this review addresses ERα, exclusively, hereafter ER refers to only ERα.

When activated by estrogen binding, ER dissociates from heat shock protein, dimerizes, translocates into the nucleus, and recruits coregulators to the regulatory regions in the target genes to modulate gene transcription. ER coregulators have been shown with diverse functions which include acetylation, methylation, ubiquitination and phosphorylation [8].

ER protein is assayed in breast cancer because it is a clinically useful prognostic factor and is predictive of response to endocrine therapy. A majority of newly diagnosed breast cancers express levels of ER protein that are clinically detectable either by immunohistochemistry or by cytosolic ligand-binding assay. In the past, ligand-binding assay was used to examine the level of ER in breast tumors. Tumors with an ER content of $\geq$10 fmol/mg protein were considered to be ER-positive [9]. Immunohistochemistry is less costly and is now more widely used to assess ER status in breast tumors which can predict response to endocrine therapy, although the ER status determined by immunohistochemistry is not always in agreement with the ligand-binding assay method [10]. Tumors that show detectable ER protein is at least 10% of tumor nuclei are designated ER positive. About two thirds of newly diagnosed breast cancers are ER positive and one third are ER negative. Endocrine therapies utilized in breast cancer care oppose estrogen action and are comprised of either ER-blocking agents (tamoxifen, raloxifene) or aromatase inhibitors (letrozole, anastrozole or exemestane). These are used to prevent breast cancer development or recurrence, or to treat metastatic disease [7, 11].

2 Mechanisms Underlying ER Loss in Breast Cancer

ER negative breast cancers have a worse prognosis and are resistant to antiestrogen therapy [7]. While estrogen is a mitogen for cultured ER positive breast cancer cell lines and primary ER positive cancers, the proliferation of ER negative breast cancer has been thought to be estrogen independent. This conclusion has been based on the observations that ER negative cancers do not respond to therapeutic ER blockade [12, 13] and that, when grown in tissue culture, ER-negative breast cancer lines do not require the presence of estrogens to sustain proliferation and are thus, estrogen independent for growth. The mechanisms underlying the lack of ER protein expression in these breast cancers is not entirely clear and appears to be multifactorial.

2.1 ER Gene Changes

Homozygous deletion of the ER locus on chromosome 6q has not been reported in breast cancers and loss of homozygosity (hemizygous loss) at 6q affects ER positive and negative cancers equally [14, 15]. ER gene mutations are relatively uncommon. A study of 200 primary breast cancers revealed few polymorphisms and only one ER mutation in an ER negative cancer [16]. Thus, ER gene changes are too uncommon to account for ER negative breast cancer [15, 16].

2.2 ER Promoter Hypermethylation

ER promoter hypermethylation was observed in six ER negative lines and demethylating agents restored ER mRNA expression [17, 18]. However, ER promoter methylation was detected in only a small portion of primary ER negative breast cancers examined (in nine of thirty nine cases or 23%) [19]. Indeed a comprehensive analysis of large number of primary breast cancers has yet to be done and the true frequency of ER hypermethylation in breast cancers is not established. Histone deacetylase inhibitors (trichostatin A) and 5-aza-2′-deoxycytidine have been shown to restore ER mRNA expression and ER protein levels in ER negative breast cancer lines [20, 21], raising the provocative possibility that histone deacetylase inhibitor drugs may have value in converting some ER-negative cancers to ER-positive, opening the possibility of this therapy to restore ER expression and anti-estrogen responsiveness [22]. This has led to the development of clinical trials for HDAC inhibitors in ER negative breast cancer, but these are still in clinical development.

2.3 ER mRNA Expression in Breast Cancers

Three early studies, using relatively insensitive non-quantitative dot blot, Northern and PCR showed a majority (43/64 assayed) of ER negative cancers express ER mRNA [23–25]. With the development of more sensitive and quantitative techniques, quantitative real-time PCR detected ER mRNA in all of 56 ER negative cancers [26, 27]. ER positive tumors tended to have higher ER mRNA levels, with significant overlap in ER mRNA values between ER positive and negative [26, 27]. ER promoter methylation may account for the lowest ER mRNA levels observed [27]. Our highly sensitive real-time PCR quantitation showed ER mRNA expression in all of 250 primary breast cancers assayed, with high variability and overlap in concentrations of ER mRNA between ER positive and negative [28]. We also observed a trend to higher ER mRNA in the ER positive cancers.

Although microarray studies have shown reduced ER gene expression in ER negative breast cancer [29–31], in these studies, individual breast cancer ER mRNA was compared to a reference of pooled cRNAs from ER positive and negative tumors [29] or to the average signal from all tumors [30, 31]. These findings are thus consistent with RT-PCR data showing ER mRNA in all breast cancers. Other array studies show variable ER [32]. QPCR from fixed paraffin embedded tissues using the Oncotype Dx analysis also indicate lower ER mRNA in ER- cancers [33–35]. However, the expression array types of analysis exhibit only about five fold variability in ER mRNA levels while QPCR from fresh frozen tissue yields up to seven logs variability in ER mRNA levels and higher sensitivity. It is noteworthy that other QPCR analysis using paraffin embedded breast cancer samples also revealed ER mRNA detection in ER negative tumors. Ma et al. [36] also showed ER mRNA values overlap between ER positive and negative tumors in over 800 primary breast cancers with lower values in the ER negative. Since highly sensitive real-time PCR shows uniform expression with variable and overlapping ER mRNA levels in ER positive and ER negative primary breast cancers, post-transcriptional and/or post-translational control of ER may also play a role in regulating ER protein levels in breast tumors [26–28].

2.4 MAPK Activated Loss of ER Expression

Recent work has implicated activation of several oncogenes upstream of MAPK in the loss of ER expression in breast cancers. El-Ashry's group developed MCF-7-derived models with inducible EGFR [37], and constitutively active (ca) c-erbB-2 [38], c-Raf1 [39], and MEK1 [40] and showed that activation of these EGFR and erbB-2 effectors decreased levels of ER and caused estrogen-independent growth [40]. SiRNA to MAPK restored ER levels in these lines, indicating that MAPK activation is causally linked to ER loss and MAPK may mediate ER negativity in at least a subset of tumors with EGFR or erbB-2

overexpression [41]. In three other established ER- breast cancer cell lines, SUM 229 (high EGFR), SUM 190 (high EGFR and erbB-2), and SUM 149 (high RhoC and EGFR), MAPK inhibition by MEK inhibitor U0126 also increased ER [42]. This mechanism appears relevant to cells that may have initially expressed high ER protein and RNA levels, but in which oncogenic activation of MAPK arises during malignant progression. This mechanism involved both ER protein and later RNA loss and arises during long term estrogen deprivation in vitro. Recent work has identified that the ER can be targeted by miRNA 222 and this was shown to be overexpressed more frequently in ER negative than ER positive breast cancers in a limited retrospective analysis [43]. The extent to which this underlies ER negative breast cancers is yet to be defined. MAPK has been shown to upregulate miRNA 222 and may underlie the MEK/MAPK mediated ER loss (El-Ashry et al., unpublished).

3 EGFR Family and Src Kinase Activation in Breast Cancer

EGFR family activation is strongly linked to ER negative breast cancer. Different studies showed the ErbB2/Her2 gene is amplified [44] and EGFR over-expressed [45] in up to 30% of primary invasive breast cancers. Both are associated with poor prognosis [45] and ER negativity in primary breast cancers [46, 47]. EGFR activation is frequent in triple negative breast cancers [48]. EGFR and erbB2 activate the Raf/MEK/MAPK pathway. The MAPK pathway is often hyperactivated in breast cancers compared to benign tissue [49], due to activation of upstream regulators, Raf-1 and MEK. MAPK hyperactivation is more frequent in ER negative compared to ER positive breast cancer. EGFR and ErbB2/Her2 each bind Src to catalyze mutual kinase activation and stimulate cell proliferation [50].

The first non-receptor tyrosine kinase identified was the v-Src oncogenic protein which plays a role in oncogenesis [51]. The vertebrate counterpart of v-Src, c-Src was identified shortly after [52]. It belongs to a family of closely related non-receptor tyrosine kinases called Src family kinases that include Src, Yes, Fyn, Fgr, Lck, Lyn, Hck, and Blk. They are closely related with a wide variety of functionality depending on cell type and cell growth. They can be involved in signal transduction, cellular proliferation, migration, differentiation and transformation [53, 54]. Src, Yes and Fyn are ubiquitously expressed in many human tissues [55]. Others are mainly expressed in hemopoietic tissues. Of these, Src is the best studied and is known to be deregulated in multiple tumor types, including breast, prostate, lung and pancreatic cancers [56].

Src is a 60 kDa tyrosine kinase and is the best-studied member of Src family kinases. Src-deficient mammary epithelial cells have been shown to have impairment of signaling pathways in response to estrogen, suggesting Src plays a role in ER signaling in vivo [57]. Src expression and or activity is elevated in many

different epithelial cancers, including breast and ovarian cancers [58–66]. Our recent immunohistochemical analysis of activated Src used a phospho-specific antibody (pY416-Src) in 482 tumors. Of these approximately 39% showed strong Src activation. ER negative status was strongly correlated with Src activation ($p = 0.002$) (unpublished data and [67]. The increased levels and activation of Src in human breast cancer provide a rationale for targeting Src in breast cancer [68]. Src specific inhibitor as a single agent to treat the breast cancer showed modest activity. The trials of combination with other agents are ongoing [69].

4 ER Cross-Talk with Signaling Transduction Pathways

In addition to genomic function, which modulates ER target gene transcription, ER also plays a role in the rapid transient actions of estrogen that do not require gene expression and have been termed non-genomic action. While the highest steady state levels are detected in the cell nucleus, there is evidence that some of the regulatory actions of ER may be extranuclear. Liganded ER rapidly and transiently activates Src and Shc, leading to Ras/MAPK as well as PI3K/AKT activation (see Fig. 1) [70–72]. Indeed recent elegant work has indicated that liganded ER is recruited to the cell surface via interaction with the cytoplasmic portion of the IGF-1R [73, 74]. ER interaction with Src is modulated by Src interacting proteins, MNAR/PELP1 and p130CAS [75–77].

The activation of signal transduction pathways by the cross-talk with liganded ER leads to ER phosphorylation at multiple sites by various kinases. ER is predominantly phosphorylated on S118 by MAPK [78], and to a lesser extent on S104 and S106 by CyclinA-CDK2 [79]. S167 may be phosphorylated by RSK1 or AKT [80, 81]. These phosphorylation events all affect the N-terminal region of ER which contains ligand independent activation function 1. PKA has been shown to phosphorylate S236, which is in the DNA-binding domain [82], and S305, which is at the start of ligand binding domain (LBD) [83]. These phosphorylation events appear to modulate ER function by altering binding to ligand, promoter DNA binding, and ER coactivators [84].

Even in the absence of estrogen, ER can be activated by several growth factors through activated receptor tyrosine kinases like EGFR and IGF-1R, which also activate Src, MAPK, PI3K/AKT pathways and lead to ER phosphorylation [85–88].

5 ER Phosphorylation by Src

Tyrosine phosphorylation of the ER has been implied the earliest in ER signaling [89, 90] and is stimulated by estrogen [91]. Early work indicated that ER-Tyrosine 537 (Y537) can be phosphorylated by Src [92]. However this was for years

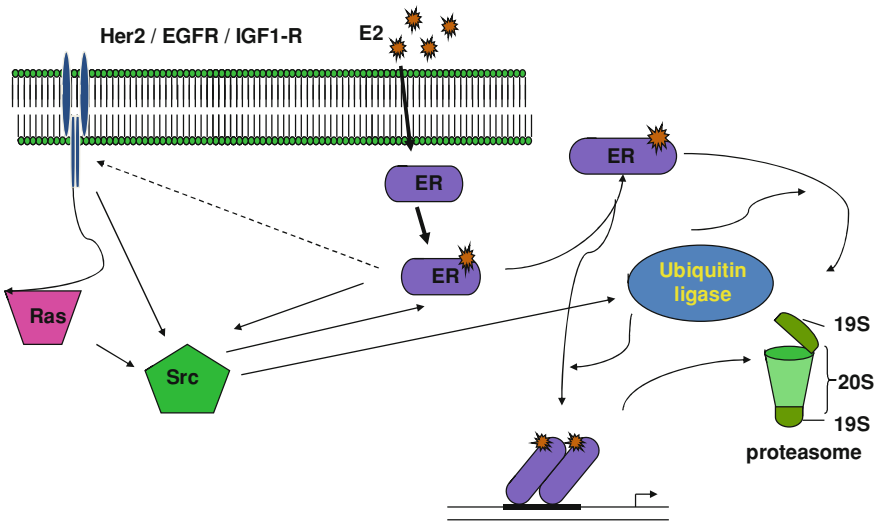

Fig. 1 Liganded ER and Src recruit proteins that serve dual roles as coactivator/ubiquitin ligases to couple ER target gene activation with ER proteolysis. This would serve to facilitate ER removal and recharging of the promoter once fired and fine tune receptor transcriptional activity. In addition, in the context of high levels of EGF and IGF-1R activation, high constitutive Src family action may permit rapid turnover of the ER in a context in which estrogen and Src are constitutively driving ER transcriptional activation. In tissues in which hormone stimulates rapid cell growth such as the uterus, ovary and breast epithelium this scenario may occur in the context of low steady state ER levels

considered controversial and a role for this phosphorylation event in ER action was not known. There are twenty-three tyrosine residues in the full length human ER. While multiple tyrosine sites in ER could be potential Src targets, in vitro Src kinase reaction generate phosphorylation of on average about two tyrosine sites per ER molecule and Y537 is one of these major sites [93]. Using a phosphorylation site prediction program [84], our analysis showed Y537 to be the single site mostly likely to be phosphorylated by Src among five tyrosine residues in the ER LBD, based on estradiol/ER LBD structure [94], consistent with early experimental results [95]. Tyrosine phosphorylation of the ER increases its affinity for estradiol [93]. A peptide containing the sequences around the phosphotyrosine residue Y537 in ER can block the ER/Src interaction and cell growth stimulated by estrogen [96]. Src also affects activation function 1 of ER [97]. Recent data indicated two additional tyrosine residues in the amino-terminal half of ER, Y52 and Y219 can be phosphorylated by Abl non-receptor tyrosine kinase in vitro. Those two may also be Src targets in ER at its amino- terminus [98]. Phosphorylation of Y537 could potentially affect ER coactivator binding, ER degradation as well as ER transactivation. This notion is supported by recent work from our lab [99].

6 The Link Between Steroid Hormone Receptor Activation and Receptor Degradation

The ubiquitin-proteasome pathway regulates eukaryotic gene transcription in a number of important ways. For many transcription factors the very phosphorylation events and protein–protein interactions that stimulate their transcriptional activity also trigger factor proteolysis [100–102]. Signaling pathways that activate many transcription factors, including NFκB, c-Jun, c-Myc, Gcn4, and E2F-1 also trigger their ubiquitin dependent degradation [100]. Components of the basal transcription apparatus can phosphorylate and activate transcription factor proteolysis [100]. Ubiquitin-mediated degradation can efficiently limit transactivator availability and action [103, 104]. In addition, ubiquitylation is required for the activity of certain transcription factors [100, 105] and may influence co-activator binding [100]. Co-activators can also enhance transcription factor ubiquitylation [100–102, 106].

Ligand mediated proteolysis regulates the turnover of most nuclear hormone receptors (NHR) including progesterone [107], thyroid hormone [108], retinoic X [109] and estrogen receptors [110–112]. The magnitude and duration of NHR transcriptional activity is also regulated by the ubiquitin proteasome pathway. Many ubiquitin proteasome components are co-activators of steroid hormone receptors [113], including the ubiquitin ligases E6AP [114], receptor potentiation factor 1/reverse Spt phenotype 5 (RPF1/RSP5) [115], MDM2 [116, 117], and BRCA1 [118, 119]; the sumo-conjugating enzyme ubc9 [120, 121]; and the 19S proteasomal subunit, yeast suppressor of gal1/thyroid receptor interacting protein 1(SUG1/TRIP1/rpt6) [122]. Overexpression of the ubiquitin ligase component NEDD8 can impair ER transcriptional activity [123, 124]. Several E2-Ubcs also regulate the levels and activities of NHR co-activators [125] and ubiquitin conjugating enzyme UbcH7 can itself act as a steroid receptor coactivator [125, 126]. Thus, the proteasome pathway can facilitate co-repressor/coactivator exchange and transcription complex remodeling [113, 125, 127].

7 Src Promotes Ligand Activated ER Degradation and ER Target Gene Transcription

Cellular ER protein levels are delicately regulated [128]. Estrogen binding to ER not only activates ER transactivation, but also leads to ubiquitin-dependent ER proteolysis [112, 129, 130]. Certain ubiquitin ligases have been identified as ER coactivators, including E6AP [114], MDM2 [116], and BRCA1 [118, 119]. The binding of these E3 ligase/coactivators may regulate both ER transcriptional activation and its proteolysis. Paradoxically, proteasome inhibition decreases ER

transcriptional activity at some ER target promoters, despite an increase in ER protein levels [130].

As noted above, the phosphorylation-dependent activation of many transcription factors is linked to their proteolysis. Many ubiquitin ligases recognize and bind only appropriately phosphorylated substrates to facilitate their ubiquitylation and proteolysis [131]. Substrate phosphorylation is usually tightly regulated to ensure the proper timing and extent of its recognition by the ubiquitin ligase that mediates its proteolysis. Specific phosphorylation event that trigger proteasomal degradation has been identified for progesterone receptor which is a member of nuclear receptor superfamily [132].

We have found that Src regulates ER transcriptional activity and also its proteolysis. Tyrosine phosphorylation of ER by Src in vitro increases ER ubiquitylation and 26S proteasome mediated ER degradation. In vivo, Src inhibitor PP1 impairs estrogen stimulated ER ubiquitylation. We have constructed MCF-7 human breast cancer cell line with induced expression of constitutive active Src. Estrogen stimulated ER proteolysis was accelerated when Src expression was induced. At the same time, estrogen stimulated ER target gene expression, like GREB1 and pS2, was elevated. Among 101 primary breast tumors tested, Src and ER levels were inversely correlated. In ER negative BT-20 cell line, ER protein was detected although at a very low levels in proliferating cells, but increased when cells were deprived of estrogen, and Src knockdown increased ER levels [29].

The mammary tissue of E6AP null mouse shows increased ER protein compared to wild-type littermates. The transgenic mouse which over expresses E6AP in mammary tissue has reduced ER protein level [133]. We recently also show that E6AP can act as ubiquitin ligase for ER in vitro and E6AP-mediated ER ubiquitylation was increased by pre-treatment of ER with Src. ER-phosphorylation by Src at Y537 enhances ER recognition by E6AP and promotes both ER proteolysis and ER target gene transcription [99].

8 Implications for the Definition of an "Estrogen Responsive" Tissue

The data above and increasing data in the field support a model in which liganded and/or appropriately phosphorylated ER recruits co-activators that include ubiquitin conjugating enzymes and ubiquitin ligase components to promote not only transcriptional activation of certain target genes, but also ER degradation. Our findings indicate that Src plays an active role in ER signaling and that ER activity may not be solely dependent on the steady state level of ER protein. This mechanism of coupled ER activation and proteolysis may be at play in a number of hormonally regulated cancers, including ER "negative" breast, ovarian and endometrial cancers, certain forms of colon cancer and malignancies of bone and

brain. Thus one could conceive of tumor tissues, and indeed of scenarios during rapid growth factor receptor and steroid stimulated proliferation of normal hormone responsive tissues in which low steady state levels of receptor are present, but the receptor itself is disproportionately activated, such as would be the case with rapid turnover of ER when Src is highly active. These data permit the possibility that a subset of ER "negative" breast tumors and indeed certain states of hormonally regulated normal tissue growth may prove to be estrogen regulated: they express ER mRNA, but ER protein levels are low or undetectable due to accelerated ligand and Src mediated ER proteolysis.

Liganded receptor cross talk with different signaling kinases, including Src, may predicate promoter selection and occupancy in the presence of estrogen in different tissue contexts. As we explore the relationship between steroid receptor turnover and transcriptional activation, we may find ways in which different cross talk-mediated receptor phosphorylation events drive differences in broad patterns of target gene expression, coactivator or repressor binding and chromatin conformational changes in the presence of various activated signal transduction pathways that are germane to receptor action at low to undetectable receptor levels. There may indeed be situations in hormone driven normal and malignant tissues where receptor levels are present at vanishingly low levels, but receptor driven transcriptional activation brisk. These concepts open a new way of viewing hormone sensitive physiology in tissue with low to undetectable hormone receptor levels.

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Appendix 4 Zubrod abstract 2013

ER, SKP2 and E2F-1 form a feed forward loop driving late ER targets and G1 cell cycle progression.

Wen Zhou[1,2], Satish Srinivasan[2], Zafar Nawaz[1,2], Joyce M. Slingerland[1,2,3].
[1]Braman Family Breast Cancer Institute, University of Miami Sylvester Comprehensive Cancer Center, [2]Departments of Biochemistry & Molecular Biology and [3]Medicine, University of Miami Miller School of Medicine, Miami, FL 33136, USA

For many transcription factors, activation is linked to degradation. This is also true for estrogen receptor α (ERα): estrogen stimulation activates ERα proteolysis. Given the importance of estrogen as a driver of breast cancer progression, we investigated mechanisms governing ERα proteolysis and how this may be linked to estrogen driven gene expression.

S-phase kinase-associated protein 2 (SKP2) is an F box component of a multi-protein SCF ubiquitin ligase that acts on multiple substrates. Levels of SKP2 have been reported to be inversely related with ERα levels in breast cancer. We also identified SKP2 as a late-acting coactivator that drives ERα targets to promote G1-to-S progression.

Our data support a model that estrogen activated ER ubiquitylation may be mediated by members of the SCF-family of ubiquitin ligases.

Appendix 5 Nature Miami 2013 Winter Symposium abstract

The SCF F box protein, SKP2, is a novel estrogen receptor α dual-role coactivator that affects cancer cell progression.

Wen Zhou[1,2], and Joyce M. Slingerland[1,2,3]

[1]Braman Family Breast Cancer Institute, University of Miami Sylvester Comprehensive Cancer Center, [2]Departments of Biochemistry & Molecular Biology and 3Medicine, University of Miami Miller School of Medicine, Miami, FL 33136, USA

Estrogens regulate key features of metabolism such as food intake, body weight, glucose homeostasis/insulin sensitivity, body fat distribution, lipolysis/lipogenesis, inflammation, locomotor activity, energy expenditure, reproduction, and cognition. Diminished ERα activity is associated with obesity in both women and men, but mechanisms thereof remain obscure. Previous reports focus on nongenomic signaling of ER in metabolism, yet our present data link estrogen:ERα-driven transcription with cell metabolism (classic or genomic signaling). While liganded ERα induces many genes in 1-4 hours, gene activation >6 hours is thought to be indirect. Here, we identify SKP2 as a late-acting coactivator that directly drives ERα targets progression. Estrogen-activated CyclinE-CDK2 binds and phosphorylates ERαS341, to prime ERα-SCFSKP2 binding via SKP2-L248QTLL252 in late G1. Of the putative late ERα targets identified by expression profiling, some of these genes are important metabolic regulators involved in cancer cell survival. Ongoing biochemical and genetic techniques will investigate the importance that these late E2-target genes in affecting cancer cell progression.

Appendix 6 FASEB 2012 abstract

The SCF F box protein, SKP-2, is a key component of an E3 ubiqutin ligase that governs Estrogen Receptor α stability

Wen Zhou[1,2], Jun Sun[1], Satish Srinivasan[1,2], Zafar Nawaz[1,2] and Joyce M. Slingerland[1,2,3]
[1]Braman Family Breast Cancer Institute, University of Miami Sylvester Comprehensive Cancer Center, [2]Departments of Biochemistry & Molecular Biology and [3]Medicine, University of Miami Miller School of Medicine, Miami, FL 33136, USA

Breast carcinoma is the most common cancer among women in developed countries, and about 70% of these tumors express estrogen receptor (ER). ER is a transcription factor and master regulator of estrogen stimulated proliferation and its expression indicates potential for response to estrogenic stimulation. A majority of ER positive breast cancers initially respond well to selective estrogen receptor modulators (such as tamoxifen) or to aromatase inhibitors.

Understanding the regulation of ER levels and its role in transcription of estrogen driven genes is thus highly germane to breast cancer therapy. There is evidence that for many transcription factors, activation is linked to factor degradation. Moreover, estrogen stimulation is known to activate ER proteolysis. Given the importance of estrogen as a driver of breast cancer progression, we have investigated mechanisms governing ER proteolysis. S phase kinase-associated protein 2 (SKP2) is an F box component of a multiprotein SCF ubiquitin ligase that acts on multiple substrates. Levels of SKP2 have been reported to be inversely related with the levels of ER in breast cancer. Here we report that SCFSKP2, comprised of SKP2, in association with Skp1, Cul1 and Rbx1, is a ubiquitin E3 ligase for ER. Ectopic expression of dominant negative Cul1 increases ER levels by impeding ER degradation in breast cancer cells. Knockdown of SKP2 impairs estrogen-triggered ER proteolysis, while ectopic SKP2 expression decreased ER stability. We show that SKP2, Skp1, Rbx1 and Cul1 co-precipitate with cellular ER and the formation of this ER/SCFSKP2 complex is cell cycle regulated and parallels CDK2 activation. We also show ER is an in vitro substrate that is ubiquitylated and degraded by SCFSKP2. The involvement of CDK2-dependent ER phosphorylation in estrogen activated ER/SCFSKP2 binding, ER proteolysis and the functional consequences of this on ER transcriptional activity are under investigation. These data suggest that SKP2 plays an important role in the regulation of ER stability, and potentially in the biologic action of this key steroid hormone receptor in breast cancer.

Appendix 7 Zubrod 2012 abstract

The SCF F Box Protein, Skp-2, is a Key Component of an E3 Ubiqutin Ligase thatGoverns Estrogen Receptor α Stability

W. Zhou1,2, J. Sun1 , S. Srinivasan1,2, Z. Nawaz1,2 and J.M. Slingerland1,2,3
1Braman Family Breast Cancer Institute, University of Miami Sylvester Comprehensive Cancer Center, 2Departments of Biochemistry & Molecular Biology and 3Medicine, University of Miami Miller School of Medicine, Miami, FL

For many transcription factors, activation is linked to degradation. This is also true for estrogen receptor ERα: estrogen stimulation activates ERα proteolysis. Given the importance of estrogen as a driver of breast cancer progression, we investigated mechanisms governing ERα proteolysis and how this may be linked to estrogen driven gene expression. S phase kinaseassociated protein 2 (SKP2) is an F box component of a multi-protein SCF ubiquitin ligase that acts on multiple substrates. Levels of SKP2 have been reported to be inversely related with ERα levels in breast cancer. Here we report that SCF^{SKP2} (comprised of SKP2, SKP1, CUL1 and RBX1) is a ubiquitin E3 ligase for ERα. Ectopic expression of dominant negative Cul1 increases ER levels by impeding ERα degradation in breast cancer cells. Ectopic SKP2 expression decreased ERα stability, while SKP2 knockdown impairs estrogen-triggered ERα proteolysis, while. Each of SKP2, Skp1, Rbx1 and Cul1 co-precipitates with cellular ERα and the formation of this ER/SCFSKP2 complex is cell cycle regulated and parallels CDK2 activation in late G1. We show ER is ubiquitylated and degraded in vitro by SCF^{SKP2}. Early evidence suggests that CDK2-dependent ERα phosphorylation promotes estrogen activated ERα/SCF^{SKP2} binding to promote ERα proteolysis. The functional consequences of this on ERα transcriptional target selection are under investigation. These data suggest that SKP2 importantly regulates ERα stability, and biologic action of this steroid receptor in on gene targets expressed in late G1 that govern S phase progression.

Appendix 8 AACR 2012 abstract

Cancer Research: April 15, 2012; Volume 72, Issue 8, Supplement 1 doi: 10.1158/1538-7445.AM2012-952 Proceedings: AACR 103rd Annual Meeting
2012-- Mar 31-Apr 4, 2012; Chicago, IL

Poster Presentations - Steroid Hormone Receptor and Growth Factor Actions in Cancer 1
Abstract 952: The SCF F box protein, SKP2, is a key component of an E3 ubiqutin ligase that governs estrogen receptor α stability

Wen Zhou[1], Jun Sun[1], and Joyce M. Slingerland[1]
[1]Univ. of Miami Miller School of Medicine, Miami, FL

Breast carcinoma is the most common cancer among women in developed countries, and about 70% of these tumors express estrogen receptor (ERα). ERα is a transcription factor and master regulator of estrogen stimulated proliferation and its expression indicates potential for response to estrogenic stimulation. A majority of ERα positive breast cancers initially respond well to selective estrogen receptor modulators (such as tamoxifen) or to aromatase inhibitors.

Understanding the regulation of ERα levels and its role in transcription of estrogen driven genes is thus highy germane to breast cancer therapy. There is evidence that for many transcription factors, activation is linked to factor degradation. Moreover, estrogen stimulation is known to activate ERα proteolysis. Given the importance of estrogen as a driver of breast cancer progression, we have investigated mechanisms governing ERα proteolysis. S phase kinaseassociated protein 2 (SKP2) is an F box component of a multi-protein SCF ubiquitin ligase that acts on multiple substrates. Levels of SKP2 have been reported to be inversely related with the levels of ERα in breast cancer. Here we report that SCFSKP2, comprised of SKP2, in association with Skp1, Cul1 and Rbx1, is an E3 ubiquitin ligase for ERα. Ectopic expression of dominant negative Cul1 increases ERα levels by impeding ERα degradation in breast cancer cells. Knockdown of SKP2 impairs estrogen-triggered ERα proteolysis, while ectopic SKP2 expression decreased ERα stability. We show that SKP2, Skp1, Rbx1 and Cul1 co-precipitate with cellular ER α and the formation of this ERα/SCFSKP2 complex is cell cycle regulated and parallels CDK2 activation. We also show ER is an in vitro substrate that is ubiquitylated and degraded by SCFSKP2. The involvement of CDK2-dependent ERα phosphorylation in estrogen activated ERα/SCFSKP2 binding, ERα proteolysis and the functional consequences of this on ERα transcriptional activity are under investigation. These data suggest that SKP2 plays an important role in the regulation of ERα stability, and potentially in the biologic action of this key steroid hormone receptor in breast cancer.

Appendix 9 Curriculum Vitae

Summary

- Trained physician-scientist
- Principle Investigator of a pre-doctoral DOD award on breast cancer research
- First or co-author on 13 peer-reviewed scientific papers in journals such as Nat Rev Cancer, Oncogene, JBC and MCB
- Co-authored 2 book chapters on cancer therapy and epigenetics
- Background in Transcriptional Regulation, Cell Cycle, Protein Ubiquitylation and Post-translational Modification, ubiquitin E3 ligase, estrogen receptor, p53, DNA Damage & Repair, DNA methylation, Histone modification & remodeling as well as in Yeast Biology

Education

Columbia University, New York, NY **2014**
Postdoctoral Associate in Biological Sciences
Advisor: Dr. Carol Prives

University of Miami, Miller School of Medicine, Miami, FL **2008-2014**
Ph.D. candidate in Biochemsitry and Molecular Biology
Advisor: Dr. Joyce M. Slingerland

Peking University, School of Medicine, Beijing, China **2001-2006**
MBBS, Medical Doctor (MD) Equivalent
Hospital Residency, **Capital Institute of Pediatrics,** Beijing, China **2005**
Hospital Residency, **Jishuitan Hospital-**the 4th Affiliated Hospital of Peking University, Beijing, China **2004**
Medical curriculum **2002-2006**
Pre-Medical curriculum **2001-2002**

Professional and Teaching Experience

Graduate student expert, Howard Hughes Medical Institute NEXUS program **2013**

Hospital Observership, General Surgery, University of Miami, Miller School of Medicine, Miami, FL **2013**

Adjunct Faculty, co-hosted by **the Ministry of Health of China** and **Peking University** **2005**

- 4th National Medical Mol. Biol. Tech. Training Class, Class No. 2005-02-02-005
- Taught 40 trainees (assistant to associate professor level from nation-wide universities) DNA methylation detections, such as Bisulfite Genomic Sequencing, Methylation Specific-PCR, and CoBRA (combined bisulfite restriction assay).

Honors and Professional Membership

2010-2013 Department of Defense (DOD) Breast Cancer Research Predoctoral Traineeship Award (120k/3 years)

Jan 2014 **Travel Award,** Keystone Symposia Conferences 2014 "Nuclear Receptors: Biological Networks, Genome Dynamics and Disease" (A3), Taos, NM

Jan 2014 **Student Award,** Elsevier Miami 2014 Winter Symposium" The Molecular Basis of Brain disorders", Miami, FL

Feb 2013 **Student Award, Nature Biotech Miami 2013 Winter Symposium "The Molecular Basis of Metabolism and Nutrition", Miami, FL**

Jul 2012 **Endocrine Society Award,** FASEB Summer Research Conference: Integration of Genomic and Non-Genomic Steroid Receptor Actions, Snowmass Village, CO

Apr 2012 **U Miami MFA Award, AACR Annual Meeting 2012, Chicago, IL**

Feb 2012 **Student Award, Nature Biotech Miami 2012 Winter Symposium "Nanotechnology in Biomedicine"**

Feb 2011 **Student Award, Nature Biotech Miami 2011 Winter Symposium "Epigenetics in Development and Disease"**

Aug 2010 **FASEB Presidential Award,** FASEB Summer Research Conference, Snowmass Village, CO

Feb 2010 **Student Award, Nature Biotech Miami 2010 Winter Symposium "Targeting Cancer Invasion and Metastasis"**

Jan 2009 **Student Award, Nature Biotech Miami 2009 Winter Symposium "Interpreting the Human Genome"**

Oct 2006 **Travel Award,** the 4th Chinese Conference on Oncology (CCO), Tianjin, China

Jun 2005 **Student Award,** Annual Conference of Beijing Society of Biochemistryand Molecular Biology, Beijing

Scientific membership: Member, Endocrine Society
Member, American Society of Clinical Oncology (ASCO)
Member, American Association of Cancer Research (AACR)
Member, American Association for the Advancement of Science (AAAS)

Editorial Acitivity

Review Editor, Frontiers in Endocrinology (Cancer endocrinology section)
Review Editor, Frontiers in Oncology
Guest editor, Journal of Cellular and Molecular Biology

Ad Hoc reviewer, Acta Biochimica et Biophysica Sinica
Ad Hoc reviewer, Acta Biochimica Polonica
Ad Hoc reviewer, Acta Naturae
Ad Hoc reviewer, American Journal of Chinese Medicine
Ad Hoc reviewer, Molecular Biology Reports
Ad Hoc reviewer, Bioscience Reports
Ad Hoc reviewer, Breast Cancer (auckl)
Ad Hoc reviewer, Cell & Bioscience
Ad Hoc reviewer, Cellular and Molecular Life Sciences

Ad Hoc reviewer, Chemical Biology & Drug Design
Ad Hoc reviewer, Chromosome Research
Ad Hoc reviewer, Chromosoma
Ad Hoc reviewer, Current Medicinal Chemistry
Ad Hoc reviewer, Epigenetics
Ad Hoc reviewer, FASEB Journal
Ad Hoc reviewer, FEBS Letters
Ad Hoc reviewer, International Journal of Biochemistry and Cell Biology
Ad Hoc reviewer, International Journal of Peptide Research and Therapeutics
Ad Hoc reviewer, IUBMB Life
Ad Hoc reviewer, Journal of Molecular Biology
Ad Hoc reviewer, Protein & Peptide Letters

Invited Speaking Engagements

1. **Zhou W.** The biochemical characterization of SKP1-CUL1-RBX1-SKP2 complex as estrogen receptor co-activator-E3 ligase. **Rockefeller University.** December 19, **2013**, New York, NY. (Invited talk)

2. **Zhou W.** The characterization of SCF^{SKP2} functions on ER both as E3 ligase and coactivator. **Columbia University.** December 17, **2013**, New York, NY. (Invited talk)

3. **Zhou W.** ERα, SKP2 and E2F-1 form a feed forward loop driving late ERα targets and G1 cell cycle progression. Children Hospital of Philadelphia (CHOP) Abramson Cancer Center. October 9, **2013**, Philadelphia, PA. (Invited talk)

4. **Zhou W.** The HDAC inhibitor depsipeptide transactivates the p53/p21 pathway by inducing DNA damage. University of Pennsylvania Smilow Translational Research Center. October 7, **2013**, Philadelphia, PA. (Invited talk)

5. **Zhou W,** Sun J, Kaliappan K, Nawaz Z, Slingerland JM. The roles of E6AP and SCF (Skp1•CUL1•F-box) in Src stimulated, estrogen activated ER proteolysis and transactivation.FASEB Summer Research Conference "The Physiology of Intergrated Nuclear and Extranuclear Steroid Signalling", August 8-13, **2010**, Snowmass Village, CO.

Publications

A. Peer-review papers (Sum of Times Cited: 318)

1. **Zhou W**, Slingerland JM. Links between steroid receptor activation and proteolysis: potential relevance to therapy of hormone regulated cancers. Nature Revs Cancer. (**2013** Nov 11 accepted and arranged for Jan **2014** issue)

2. **Zhou W**, Srinivasan S, Nawaz Z, Slingerland JM. ERα, SKP2 and E2F-1 form a feed forward loop driving late ERα targets and G1 cell cycle progression. Oncogene. **2013** Jun 17. doi: 10.1038/onc.2013.197. (Epub). (Times Cited: **2**)

3. Sun J, **Zhou W**, Kaliappan K, Nawaz Z, Slingerland JM. ERα phosphorylation at Y537 by Src triggers E6-AP-ERα binding, ERα ubiquitylation, promoter occupancy, and target gene expression. Mol Endocrinol. **2012** Sep;26(9):1567-77. (Times Cited: **1**)

4. Wang H#, **Zhou W**#, Zheng Z, Zhang P, Tu B, He Q, Zhu WG. The HDAC inhibitor depsipeptide transactivates the p53/p21 pathway by inducing DNA damage. DNA Repair (Amst). **2012** Feb 1; 11(2):146-56**.** (Times Cited: **16**) **# co-first author**

5. Horn D, **Zhou W**, Trevisson E, Al-Ali H, Harris TK, Salviati L, Barrientos A. The conserved mitochondrial twin CX9C protein Cmc2 is a Cmc1 homologue essential for cytochrome C oxidase biogenesis. J Biol Chem. **2010** May 14;285(20):15088-99. (Times Cited: **6**)

6. Yuan F#, El Hokayem J#, **Zhou W**#, Zhang Y. FANCI protein binds to DNA and interacts with FANCD2 to recognize branched structures. J Biol Chem. **2009** Sep4;284(36):24443-52. (Times Cited: **20**) **# co-first author**

7. Yuan F, Lai F, Gu L, **Zhou W**, El Hokayem J, Zhang Y. Measuring strand discontinuity-directed mismatch repair in yeast Saccharomyces cerevisiae by cell-free nuclear extracts. Methods. **2009** May;48(1):14-8. (Times Cited: **2**)

8. Yang Y, Zhao Y, Liao W, Yang J, Wu L, Zheng Z, Yu Y, **Zhou W**, Li L, Feng J, Wang H, Zhu WG. Acetylation of FoxO1 activates Bim expression to induce apoptosis in response to histone deacetylase inhibitor depsipeptide treatment. Neoplasia. **2009** Apr;11(4):313-24.(Times Cited: **19**)

9. **Zhou W**, Zhu WG. The changing face of HDAC inhibitor depsipeptide. Curr Cancer Drug Targets. **2009** Feb;9(1):91-100. Review. (Times Cited: **15**)

10. Chai G#, Li L#, **Zhou W**#, Wu L, Zhao Y, Wang D, Lu S, Yu Y, Wang H, McNutt MA, Hu YG, Chen Y, Yang Y, Wu X, Otterson GA, Zhu WG. HDAC inhibitors act with 5-aza-2'-deoxycytidine to inhibit cell proliferation by suppressing removal ofincorporated abases in lung cancer cells. PLoS One. **2008** Jun 18;3(6):e2445. (Times Cited: **28**)**# co-first author**

11. Wu LP, Wang X, Li L, Zhao Y, Lu S, Yu Y, **Zhou W**, Liu X, Yang J, Zheng Z, ZhangH, Feng J, Yang Y, Wang H, Zhu WG. Histone deacetylase inhibitor depsipeptideactivates silenced genes through decreasing both CpG and H3K9

methylation on the promoter. Mol Cell Biol. **2008** May;28(10):3219-35. (Times Cited: **58**)

12. Wang H, Zhao Y, Li L, McNutt MA, Wu L, Lu S, Yu Y, **Zhou W**, Feng J, Chai G,Yang Y, Zhu WG. An ATM- and Rad3-related (ATR) signaling pathway and aphosphorylation-acetylation cascade are involved in activation ofp53/p21$^{Waf1/Cip1}$ in response to 5-aza-2'-deoxycytidine treatment. J Biol Chem. **2008** Feb 1;283(5):2564-74. (Times Cited: **28**)

13. Zhao Y, Lu S, Wu L, Chai G, Wang H, Chen Y, Sun J, Yu Y, **Zhou W**, Zheng Q, WuM, Otterson GA, Zhu WG. Acetylation of p53 at lysine 373/382 by the histone deacetylase inhibitor depsipeptide induces expression of p21$^{Waf1/Cip1}$. Mol Cell Biol. **2006** Apr;26(7):2782-90. (Times Cited: **125**)

B.Book Chapter

1. Sun J, **Zhou W**, Nawaz Z and Slingerland JM. (**2012**) Cross Talk Between ERα and Src Signaling and Its Relevance to ER Status and Hormone Responsiveness. In "Advances in Rapid Sex-Steroid Action".G. Castoria and A. Migliaccio (eds.), Chapter DOI: 10.1007/978-1-4614-1764-4_4, Springer Science+Business Media, LLC. Book ISBN 978-1-4614-1763-7.

2. Zhu WG and **Zhou W**. (**2006**) Eukaryotic Genomic DNA Methylation.p117-144, In "Chromatin and Epigenetic Regulation". Shen YF, and Wu NH, eds., Higher Education Press, Beijing. Book ISBN 7-04-018637-3.

C. Abstract

1. **Zhou W**, Weisenburger S, Slingerland JM. The SCF F box protein, FBXL6, is a novel estrogen receptor α dual-role coactivators that affects cell cycle progression. In: Keystone Symposia Conferences 2014 "Nuclear Receptors: Biological Networks, Genome Dynamics and Disease" (A3), January 10-15, **2014**, Taos, NM. Abstract nr 3039.

2. **Zhou W**, Srinivasan S, Nawaz Z, Slingerland JM. ERα, SKP2 and E2F-1 form a feed forward loop driving late ERα targets and G1 cell cycle progression. Annual Zubrod Memorial Lecture and Cancer Research Poster Session, May 17, **2013**, Miami, FL. Abstract nr 27.

3. **Zhou W**, Slingerland JM. The SCF F box protein, SKP2, is a novel estrogen receptor α dual-role coactivators that affects cancer cell metabolism. Nature Biotech Miami 2013 Winter Symposium "The Molecular Basis of Metabolism and Nutrition", February 10-13, **2013**, Miami, FL. Abstract nr 35.

4. **Zhou W**, Sun J, Srinivasan S, Nawaz Z, Slingerland JM. SCF/SKP2 E3 ligase promotes G1/S transition by ubiqutinating and activating estrogen receptor α. FASEB Summer Research Conference "The Physiology of Intergrated Nuclear and Extranuclear Steroid Signalling", July 29-August 3, **2012**, Snowmass Village, CO. Abstract nr 25.

5. Sun J, **Zhou W**, Kaliappan K, Nawaz Z, Slingerland JM. ERα phosphorylation at Y537 by Src drives ER transcription & degradation. FASEB SRC, July 29-August 3, **2012**, Snowmass Village, CO. Abstract nr 24.

6. Kaliappan K, Xie Y, Sun J, **Zhou W**, Nawaz Z, Slingerland JM. Src mediated ligand activated estrogen receptor α proteolysis via BRCA1/BARD1 ubiquitin ligase in breast cancer. FASEB SRC, July 29-August 3, **2012**, Snowmass Village, CO. Abstract nr 23.

7. **Zhou W**, Sun J, Srinivasan S, Nawaz Z, Slingerland JM. The SCF F Box Protein, SKP2, is a Key Component of an E3 Ubiqutin Ligase that Governs Estrogen Receptor α Stability. Annual Zubrod Memorial Lecture and Cancer Research Poster Session, May 18, **2012**, Miami, FL. Abstract nr 26.

8. **Zhou W**, Sun J, Slingerland JM. The SCF F box Protein, SKP2, is a key component of an E3 ubiqutin ligase that governs estrogen receptor α stability. In: Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research; **2012** Mar 31-Apr 4; Chicago, IL. **Cancer Res** 2012;72: 952. Abstract nr 952.

9. **Zhou W**, Sun J, Kaliappan K, Nawaz Z, Slingerland JM. The roles of E6AP and SCF (Skp1•CUL1•F-box) in Src stimulated, estrogen activated ER proteolysis and transactivation. FASEB Summer Research Conference "The Physiology of Intergrated Nuclear and Extranuclear Steroid Signalling", August 8-13, **2010**, Snowmass Village, CO. Abstract nr 14.

10. Sun J, **Zhou W**, Sun J, Kaliappan K, Nawaz Z, Slingerland JM. ERα phosphorylation at Y537 by Src triggers E6-AP-ERα binding, ERα ubiquitylation, promoter occupancy and target gene expression. FASEB SRC, August 8-13, **2010**, Snowmass Village, CO. Abstract nr 13.

11. Xie Y, Sun J, **Zhou W**, Slingerland JM. Src signaling promotes ligand activated ER proteolysis. FASEB SRC, August 8-13, **2010**, Snowmass Village, CO. Abstract nr 12.

12. Xie Y, Sun J, **Zhou W,** Slingerland JM. Src signaling promotes ligand activated ER proteolysis. Annual Zubrod Memorial Lecture and Cancer Research Poster Session, May 21, **2010**, Miami, FL. Abstract nr 35.

Research Support

W81XWH-11-1-0097 (**Wen Zhou as PI**)
1/1/2011-1/31/2014
Department of Defense (DOD)

The Role of Skp1-Cull-F-box Ubiquitin Ligases in Src-Stimulated Estrogen Receptor Proteolysis and Estrogen Receptor Target Gene Expression

- To investigate the role of ubiquitin ligase SCF^{SKP2} in breast cancer cells with a focus on identifying how it may contribute to molecular mechanisms underlying estrogen receptor negativity.
- Part of the accomplished results was published at **Mol Endo (2012)** and **Oncogene (2013)**.
- An invited review for **Nat Revs Cancer (2014)** about activation coupled hormone receptor proteolysis is in press.